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MOLECULAR DETECTION OF NOROVIRUSES IN READY-TO-EAT FOODS AND FRUIT PRODUCTS

Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences:

Moleculaire detectie van norovirussen in kant-en-klare levensmiddelen en fruitproducten.

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WOORD VOORAF

Zeven hoofdstukken, 209 bladzijen, 8886 regels, 72.406 woorden of 468.547 karakters: de samenvatting van 4 jaar onderzoek vol bloed, zweet en tranen! Het is uiteraard een torenhoog cliché, maar onderzoek doe je niet alleen. Ik zou dan ook een aantal mensen willen bedanken, zonder wie dit boekje nooit geschreven zou zijn.

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Ambroos Stals
Februari, 2011

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LIST OF ABBREVIATIONS

AdV	**adenovirus**
AFNOR	**Association Française de Normalisation**
ANOVA	**analysis of variance**
BHQ	**black hole quencher**
CaCV	**canine calicivirus**
Ct	**cycle treshold**
CDC	**Centers for Disease Control and Prevention**
cDNA	**copy deoxyribonucleic acid**
CEN	**Comité Européen de Normalisation (European Committee for Standardization)**
CTAB	**Cetyl trimethylammonium bromide**
DNA	**deoxyribonucleic acid**
dsDNA	**double stranded deoxyribonucleic acid**
dTTP	**deoxythymidine triphosphate**
dUTP	**deoxyuridine triphosphate**
EFSA	**European Food Safety Authority**
EIA	**enzyme immune assay**
EM	**electron microscopy**
EV	**enterovirus**
FBVE	**Food-borne viruses in Europe network**
FCV	**feline calicivirus**
FRET	**Fluorescence Resonance Energy Transfer**
GI	**genogroup 1**
GII	**genogroup 2**
GIII	**genogroup 3**
GIV	**genogroup 4**
GV	**genogroup 5**
GITC	**guanidine isothiocyanaat**
HAV	**hepatitis A virus**
HBV	**hepatitis B virus**
HCV	**hepatitis C virus**
HEV	**hepatitis E virus**
HBGA	**histo bloodgroup antigen**

IAC	**internal amplification control**
ISO	**International Organization for Standardization**
LAMP	**Loop-mediated Isothermal Amplification**
LC480	**LightCycler 480**
MNV-1	**murine norovirus 1**
NASBA	**Nucleic Acid Sequence Based Amplification**
NFMGBQ	**Non-fluorescent minor groove binding quencher**
NoV	**norovirus**
NTC	**no template control**
NTPase	**nucleotidephosphatase**
NTP	**nucleotide triphosphate**
ORF	**open reading frame**
PBS	**phosphate buffered saline**
PC	**process control**
PCR	**Polymerase chain reaction**
PEG	**Polyethyleenglycol**
PFU	**plaque forming unit**
PV	**poliovirus**
RdRp	**RNA dependent RNA polymerase**
RNA	**ribonucleic acid**
RoV	**rotavirus**
RT	**reverse transcription**
RTC	**reverse transcription control**
RTE	**ready-to-eat**
RT-LAMP	**reverse transcriptase Loop-mediated Isothermal Amplification**
RT-PCR	**reverse transcriptase Polymerase chain reaction**
RT-PCRU	**reverse transcriptase Polymerase chain reaction units**
SaV	**sapovirus**
ssDNA	**single stranded DNA**
stdev	**standard deviation**
TBEV	**tick-borne encephalitis virus**
$TCID_{50}$	**median tissue culture infectious dose**
UNG	**Uracyl-N-glycosylase**
UV	**ultraviolet**
VP	**viral protein**
WHO	**World Health Organization**

Objectives

OBJECTIVES

Noroviruses (NoV) have increasingly been recognized as a global major cause of non-bacterial gastroenteritis, also nicknamed “winter vomiting disease” (Koopmans, 2008). The spread of this pathogen is facilitated by a low infectious dose (10 to 100 infectious virus particles) combined with a high environmental stability (Baert et al., 2009a; Teunis et al., 2008). The Norwalk viral agent (later renamed NoV genotype I.I (GI.I)) was discovered in 1972 by Kapikian and colleagues. in faecal specimens originating from a gastroenteritis outbreak in Norwalk, Ohio (Kapikian et al., 1972). Nevertheless, detection of NoV was hampered by a lack of sensitive methods until the 1990’s when the cloning and sequencing of the NoV genome cleared the way for the development of more sensitive molecular NoV detection methods.

This development lead to the estimation that NoV could be responsible for 60 % and 77 % of all gastroenteritis cases with known etiology in the USA and in Europe, respectively (Mead et al., 1999; Verhoef et al., 2009). The fraction of NoV outbreaks caused by consumption of contaminated foods is estimated to be 10 to 20 % (Kroneman et al., 2008b; Verhoef et al., 2009; Widdowson et al., 2005). In Belgium, NoV has surpassed *Salmonella* as the most important food borne pathogen in 2007, 2008 and 2009 (Baert et al., 2009b)(Nadine Botteldoorn, personal communication).

Food products can be contaminated with NoV through 2 main routes. A first transmission route occurs via pre-harvest contamination (in most cases via contact with Nov contaminated water) whereby mostly fresh produce and bivalve shellfish are involved. A second main transmission route is via (post-) harvest contamination, whereby an infected food hander or food picker is often involved. A broad range of food products can be related to the latter transmission route.

A problem when investigating NoV food borne outbreaks is that studies are mostly based on epidemiological and clinical findings data the confirmation of food as source of a NoV food borne outbreak is difficult to date. Therefore, the **first goal** of this PhD consisted of the development and evaluation of methods for detection of NoV in a broad range of food products, with an emphasis on foods involved in NoV food borne outbreaks, such as soft red fruits and ready-to-eat foods. A single method for detection of NoV in all foods was not possible, since different chemical compositions of food matrices require different approaches (Baert et al., 2008a). According to their chemical composition as well as to other features, 3 different food categories can be defined: “carbohydrate/water based foods” such as fresh produce (including soft red fruits), “fat and protein based foods” including ready-to-eat (RTE) foods such as deli sandwiches or composite meals and bivalve molluscan shellfish. Although

the latter food category is a frequent source of NoV food borne outbreaks, evaluated methods for NoV detection in shellfish have been well described and were consequently not included in this PhD.

Detection of NoV in foods is more difficult compared to most food borne bacterial pathogens. First of all, cultivation of human infective NoV is not possible thus far. Secondly, detection of NoV present at very low levels on the foods is an absolute necessity due to the low infectious dose. Therefore, (genomic material of) NoV has to be extracted from the foods and has to be detected subsequently by a molecular detection method. Due to the complexity of these methods and the possibility of inhibition or reduced detection efficiency because of the food matrix, controls are required to assure the reliability of the obtained results.

The **second goal** included the evaluation of the murine norovirus 1 (MNV-1) as control reagent at different steps throughout the NoV detection methods in soft red fruits and RTE foods. The first step to accomplish both goals was the development of a multiplex real-time RT-PCR assay for simultaneous detection of genogroup I (GI) and II (GII) NoV and for MNV-1 (chapter 2). Evaluation of this assay was performed towards (1) sensitivity of the individual (singleplex) assays, (2) the influence of the multiplex setup on the sensitivity of the individual assays and (3) the usefulness of MNV-1 as internal amplification control and as reverse transcription control. During development of the multiplex assay, contamination issues were observed (chapter 3). Since this contamination could influence reliable detection of low amounts of NoV genomic material (1 to 10 genomic copies per PCR reaction), the source of the contamination was investigated and recommendations were formulated to deal with this problem. Secondly, an elution-concentration protocol and a direct RNA extraction protocol were evaluated for NoV extraction from respectively soft red fruits and RTE foods (chapters 5 and 4). The influence of factors such as NoV levels and the simultaneous presence of multiple genogroups on the sensitivity of both methods was evaluated, while the robustness of both methods was evaluated on a broad range of food matrices. Simultaneously, the use of MNV-1 was evaluated as process control for both NoV detection protocols.

After evaluation and optimization of methods for extraction and molecular detection of NoV in soft fruits and in RTE foods, the **third goal** of this PhD consisted of a screening study of fresh produce samples for NoV presence. The aim of this study was to elucidate whether NoV presence could be linked to bacteriological quality and whether preventive screening of food products is sensible. It would also aid in answering the question if foods testing positive for NoV could form a threat to public health.

Chapter 1

Molecular detection of noroviruses in foods: literature overview

1. CHAPTER 1: MOLECULAR DETECTION OF NOROVIRUSES IN FOODS: LITERATURE OVERVIEW.

1.1. Food borne viruses

1.1.1. Introduction

Food borne viruses are enteric viruses (18 to 70 nm diameter) able to infect human hosts. They are defined by several features that result in different detection, survival and inactivation methods compared to food borne bacterial pathogens. Since (food borne) viruses need specific host cells to replicate (in contrast to bacteria), infection of the host and replication of the food borne virus occur in the intestinal tract of the host organism. Viral particles are thereafter excreted in stool or emesis, mostly in high concentrations (Atmar et al., 2008; Lee et al., 2007). Likewise to bacterial pathogens such as *Listeria monocytogenes*, some food borne viruses are able to cross the intestinal barrier and can affect other organs such as the liver, muscle or nerve tissues.

Most common food borne viruses were discovered in the 1970's (noroviruses (NoV), hepatitis A virus (HAV), rotaviruses (RoV), sapoviruses (SaV)), followed by less frequent observed food borne viruses such as hepatitis E virus (HEV) and aichiviruses in the 1980's (Balayan et al., 1983; Bishop et al., 1973; Chiba et al., 1979; Feinstone et al., 1973; Kapikian et al., 1972; Yamashita et al., 1993). Cloning of food borne virus genomes in the late 1980's and early 1990's (Cohen et al., 1987; Ketner et al., 1994; Lambden et al., 1992; Matsui et al., 1993; Tam et al., 1991; Xi et al., 1990) lead to development of assays for detection of these viruses based on molecular methods instead of the less sensitive enzyme immune assays (EIA) or electron microscopy based methods. This cloning also showed that the genome of food borne viruses consists either of single stranded RNA (ssRNA; NoV, SaV, Astrovirus, Aichivirus, HAV, HEV), dsRNA (RoV) or dsDNA (adenoviruses (AdV)). A common property of most food borne viruses is their low infectious dose as an intake of 10 to 100 infectious viral particles can be sufficient for infection (Koopmans et al., 2002; Teunis et al., 2008).

The structure of food borne viruses is quite diverse, although all have a non-enveloped protein capsid protecting a nucleic acid genome. The protein capsid of most food borne viruses appeared as a circular shape when discovered by electron microscopy, which resulted in the preliminary classification as "small round viruses" (Appleton, 1987; Caul and Appleton, 1982). Using molecular methods, these viruses were later reclassified as astroviruses, NoV, SaV, parvoviruses, etc.

Although food borne viruses cannot replicate in the environment, they are resistant to environmental stressors, such as heat, high or low pH, drying, light and UV exposure (Baert

et al., 2009a; Vasickova et al., 2010). These resistances allow most food borne viruses to pass the gastric fluids in the gut and the alkalic/proteolytic activity in the duodenum, permitting infection of the lower digestive tract.

Moreover, the high persistence allows most food borne viruses to retain their infectivity in foods such as lettuce, shellfish, frozen soft red fruits, herbs and turkey meat for periods ranging between two days up to four weeks (Bidawid et al., 2001; Butot et al., 2008; Hewitt and Greening, 2004). Survival on several surfaces in hospitals, kitchens and households has been suggested as well (Barker et al., 2004).

1.1.2. Classification based on clinical features

Food borne viruses can be organized in three classes according to their clinical features.

A **first class** of food borne viruses consists of enteric viruses causing gastroenteritis and includes NoV, RoV, SaV, enteric AdV, astroviruses and aichiviruses. Ingestion of the infectious dose of one of these viruses leads to a 10 to 51 hour incubation period, followed by manifestation of typical symptoms such as abdominal cramps, fever, watery diarrhea and other symptoms such as headaches, chills and general myalgias (Dennehy, 2000; Glass et al., 2009). The illness usually lasts for 2 to 3 days, but can last longer (4 to 6 days) in nosocomial outbreaks and among children (Lopman et al., 2004; Rockx et al., 2002). The illness is self-limiting in most cases, but fatalities related to NoV induced gastroenteritis have been reported among the elderly in nursing home facilities (Jenkins et al., 2007; Mattner et al., 2006). In rare cases, viruses from this class are able to cross the intestine-blood barrier, and can be found back in the blood of patients (Medici et al., 2010; Takanashi et al., 2009). The severity of the symptoms and the target can vary between different viruses in this class. While NoV can typically trigger gastroenteritis in people of all age, RoV and SaV are in particular infectious to children under 3 and 5 years old, respectively (Rockx et al., 2002). This is most likely caused by the characteristic higher pH of the stomach at these ages. As soon as this pH level drops to normal values (pH 2), RoV are rapidly inactivated (Leong et al., 2008; Weiss and Clark, 1985). Noteworthy, five RoV serotypes have been the most common cause of severe gastroenteritis for children aged under three years in the last 35 years (Bishop, 2009). Astroviruses are suspected to cause more gastroenteritis cases compared to NoV, but illness is less severe (Clark and McKendrick, 2004). Astroviruses are also related to younger children in most cases (Koopmans et al., 2002). Upon infection by one of these viruses, a virus shedding period can occur before and during manifestation of the clinical symptoms, but can also persist for 2 to 8 weeks after disappearance of the symptoms (Atmar et al., 2008; Stebbins, 2007). In this period, extremely high numbers of virus particles (up to 10^{10}) per gram faeces can be excreted (Atmar et al., 2008; Carter, 2005; Lee et al., 2007). This shedding can persist for more than a year in patients who are

immunocompromized (Ludwig et al., 2008). Chan et al. (2006) reported a 100-fold higher viral shedding of genogroup II (GII) NoV compared to genogroup I (GI) NoV which might facilitate transmission of GII NoV through the fecal-oral route. Immunity to these food borne viruses can be obtained in various ways. A World Health Organisation (WHO) approved vaccine against RoV has been developed with a 70 to 85 % efficacy against RoV gastroenteritis of any severity (Ruiz-Palacios et al., 2006; Vesikari et al., 1984), and grants a lifetime immunity. For NoV, only a short-term immunity of 6 to 14 weeks can be obtained after infection while Astrovirus, SaV, RoV and AdV cause infections or symptoms mainly in children and long time immunity is developed (Koopmans et al., 2002). A lower susceptibility for the Norwalk (GI.I) NoV can be observed in so-called secretor-negative individuals (20 % of the European-derived population not expressing a functional 1,2 fucosyl transferase (FUT2))(Radford et al., 2004) and people with blood type B seemed to have a reduced risk of NoV infection (Rockx et al., 2005).

A **second class** of food borne viruses includes enteric viruses that cause hepatitis (HAV (Fig 1.1) and HEV). Hepatitis is an inflammation of the liver (either acute or chronically) and is mostly caused by viral infection of Hepatitis A, B, C, D or E viruses, but only hepatitis A and E viruses are food borne viruses (Martin and Lemon, 2006). In contrast to the gastroenteritis inducing food borne viruses, manifestation of the symptoms occurs 2 to 8 weeks after ingestion of the infectious dose (Greening, 2006; Worm et al., 2002). Clinical symptoms are fever, malaise, anorexia, nausea, abdominal discomfort, dark urine and jaundice and most damage to the liver is done by the immune response of the human body rather than the viral agents (Johnston, 2010). The immune reaction of the host develops 2 to 3 weeks after infection and attacks the liver (Carter, 2005). Symptoms caused by HEV are generally more severe compared to HAV (Chau et al., 2006) and in pregnant women a case-fatality rate up to 31% has been reported (Boccia et al., 2006; Khuroo et al., 1981). HEV and HAV virus particles can be shedded 2 to 3 weeks after infection, often before the appearance of the clinical symptoms (Atkinson et al., 2000; Worm et al., 2002). The illness usually lasts no longer than two months, although individuals may have relapsing signs and symptoms up to six months. Noteworthy, young children (< 6 years of age) have significantly more chance for an asymptomatic infection (Johnston, 2010). A zoonotic reservoir for HEV has been proven, since identical strains occurred in animals and in patients (Bouwknegt et al., 2007; Tei et al., 2003). Vaccines for HAV are currently available, while a HEV vaccine is in clinical trial (Nothdurft, 2008; Panda et al., 2007).

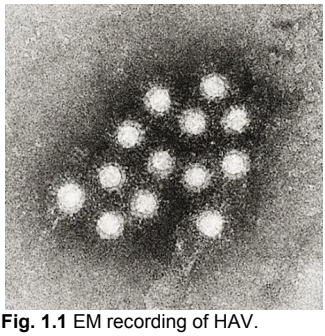

Fig. 1.1 EM recording of HAV. (source: CDC Public Health Image Library)

A **third class** of food borne viruses contains enteric viruses related to other clinical features. Coxsackie- and echoviruses (members of the *Enterovirus* genus) have caused a limited number of food borne outbreaks (Cliver, 1997). Tick borne encephalitis virus (TBEV) may also be food borne and can occasionally be transmitted by the intake of dairy products based on unpasteurized milk from viraemic livestock (Cliver, 1997; Lindquist and Vapalahti, 2008). Finally, parvoviruses have been linked with consumption of shellfish (Appleton and Pereira, 1977).

It should be noted that this list is not complete as only the most important food borne viruses were described.

1.2. Noroviruses

1.2.1. Classification

Noroviruses were discovered in 1972 by immune electron microscopy (Fig 1.2) of faecal samples of volunteers challenged with faecal filtrates from a group of elementary school students affected by an outbreak of gastroenteritis in 1968 in Norwalk, Ohio (Kapikian et al., 1972). The 27 – 32 nm viral agent was originally named Norwalk virus and was later recognized as the type agent of the genus *Norovirus* (previously denoted as "Norwalk-like viruses" or "small round structures viruses"). Together with the genera *Sapovirus* (previously called "Sapporo-like viruses"), *Lagovirus, Vesivirus* and the newly proposed *Nebovirus*, the *Norovirus* genus forms the *Caliciviridae* family (Green et al., 2000; Oliver et al., 2006).

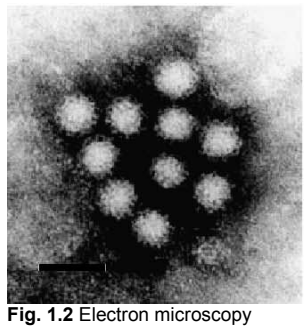

Fig. 1.2 Electron microscopy recording of NoV. (source: Koopmans and Duizer, 2004)

Characterization of the NoV genus has been troubled by the non-existence of simple immunological or biological methods (e.g. serotyping) and thus the analysis of nucleotide and amino acid sequences has become the method of choice. Initially, several authors proposed classification schemes grounded on (short) nucleotide sequences (~150 - 300 bp) in the NoV genome used for genotyping of NoV in samples (Fig 1.8). However, no consensus could be found, especially for the human infective NoV (Fankhauser et al., 2002; Kageyama et al., 2004; Vinje et al., 2004). Therefore, a classification scheme based on the amino acid sequences for the major capsid protein (VP1) of 141 NoV strains was developed, which is now widely accepted although an official taxonomy has not been approved yet for the NoV genus (Zheng et al., 2006). The amino acid sequence of the VP1 protein (530 – 555 amino acids) is encoded by the gene (~1.6 kb) situated in open reading frame (ORF) 2 of the NoV genome. In this classification, the NoV genus contains 5 genogroups whereby genogroup I and II (GI and GII) consist of 8 and 17 – extended to 19 by Wang et al. (2007) – genetic

clusters, respectively. Both genogroups contain most of the human infective NoV genotypes, together with the Alphatron and Ft. Lauerdale genotypes in genogroup IV (GIV). The latter genogroup also contains a number of NoV strains infecting carnivores such as dogs and lions (Martella et al., 2007; Martella et al., 2008). Bovine and murine NoV are classified respectively in genogroup III (GIII) and V (GV), while porcine NoV are also classified in GII.
The extensive genetic diversity within the NoV genus is most likely caused by (1) the lack of proofreading activity of the NoV RNA dependent RNA polymerase (RdRp) allowing a high mutational rate (Barr and Fearns, 2010; Cameron et al., 2009) and (2) recombination between different NoV strains (Bull et al., 2007; Mathijs et al., 2010).

1.2.2. Genome and virion structure

Molecular cloning of the NoV genome (Xi et al., 1990) lead to the understanding of the molecular virology as well as the development of molecular detection methods. The latter is a necessity, since NoV cannot be grown reliably in cell culture, despite several attempts (Asanaka et al., 2005; Duizer et al., 2004; Straub et al., 2007).
The genome of NoV consists of a 7.5 – 7.7 kb positive single stranded RNA (ssRNA) molecule with a polyadenylated 3' side. It has three open reading frames (ORFs) which encode all structural and non-structural proteins (Fig 1.3). The first ORF (ORF1; 5.1 to 5.3 kb) encodes (from 5' to 3') protein p48, protein p41 with nucleotide triphosphatase acitivity (NTPase), protein p22, VPg, $3CL^{pro}$ and RdRp, while the second (ORF2; 1.6 kb) and third ORF (ORF3; 0.6 to 0.9 kb) encode for the structural proteins VP1 and VP2, respectively (Hardy, 2005). The most conserved area in the dynamic NoV genome is considered a 102 (nucleotides 5279 to 5381; GI NoV) to 120 (nucleotides 4988 to 5108; GII NoV) nucleotide sequence which covers the sequence overlap between the first two ORFs (Kageyama et al., 2003). Although the genome is rather conserved within a genogroup, NoV strains from different genogroups have but a 51 % to 61 % nucleotide sequence similarity (Kojima et al., 2002). Moreover, the ORF2 sequence coding for the major capsid protein (VP1) can differ as much as 60 % between genogroups and up to 30 % between genotypes within a single genogroup (Donaldson et al., 2008; Donaldson et al., 2010).

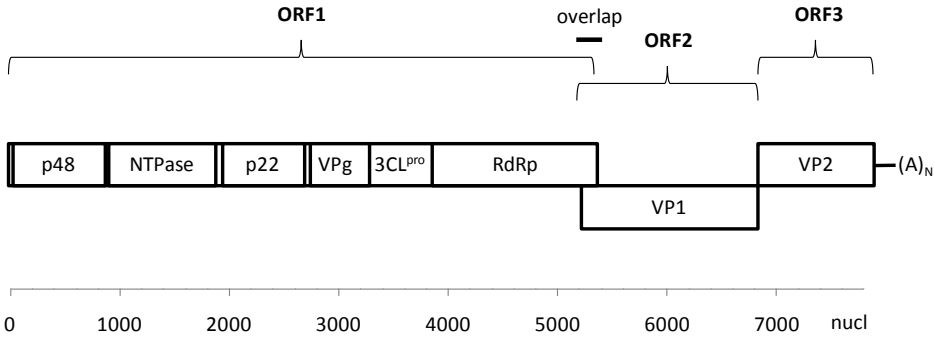

Fig. 1.3 Overview of the NoV genome organization.

The NoV virion consists of 90 dimers of VP1 that form a T=3 icosahedral structure and of 1 or 2 VP2 proteins. The VP1 protein contains a hypervariable P2 amino acid region responsible for receptor binding to the infected host cells (Taube et al., 2010) and a single amino acid substitution results in reduced infectivity of the murine norovirus 1 (MNV-1), a NoV surrogate virus (Bailey et al., 2008; Shirato et al., 2008). Protein structure analysis of VP2 suggests that this protein may function in the capsid packaging of the NoV genome. Studies with the feline calicivirus (FCV) have shown that VP2 interacts with VP1 during the assembly of virus particles (Di Martino and Marsilio, 2010; Glass et al., 2000). The p48 protein is involved in the disruption of intracellular protein trafficking of infected host cells by interacting with their Golgi apparatus (Ettayebi and Hardy, 2003). Protein p41 seems to have a nucleoside triphosphatase (NTPase) activity allowing binding and hydrolyzation of nucleoside triphosphates (NTPs)(Pfister and Wimmer, 2001). The VPg protein has a direct function in ribosome recruitment to the viral RNA through direct interaction with translation initiation factor eIF3 and the 40S ribosomal subunits (Daughenbaugh et al., 2003; Daughenbaugh et al., 2006; Herbert et al., 1997). The $3CL^{pro}$ protein is responsible for the cleavage of the ORF1 polyprotein in at least six proteins (Hardy, 2005). The active form of the RNA dependent RNA polymerase (RdRp) is a homodimer, and RdRp has an essential function for replication of NoV virus particles (Hogbom et al., 2009). Finally, the function of p22 has to be determined yet (Hardy, 2005).

1.2.3. NoV GII.4

The NoV genotype most commonly identified in NoV gastroenteritis outbreaks is the NoV GII.4 genotype. Therefore, its epochal evolution has been documented thoroughly between 1974 and 2007. In the USA, only 2 major NoV GII.4 strains were observed between 1974 and 1994 (a NoV GII.4 ancestor strain and GII.4 Camberwell). In the 7 year period between 1995 and 2002 a single major NoV GII.4 strain was considered dominant (NoV GII.4

Grimsby) both in Europe and in the USA. Meanwhile, less frequent detected strains such as NoV GII.4 Erfurt, GII.4a and GII.4c circulated as well in both continents. Subsequently, NoV GII.4 Farmington Hills and Hunter (also respectively named NoV GII.4 2002 and NoV GII.4 2004 in Europe) caused 2 NoV epidemic seasons in 2002-2003 and 2004-2005. Simultaneously, other NoV GII.4 strains such as GII.4 Henry (2000-2005), GII.4 Chiba (2004-2005), GII.4 Yerseke (2005-2006) and GII.4 Osaka (2005-2006) were prevalent in the USA and in Europe. Finally, NoV GII.4 Laurens and NoV GII.4 Den Haag (also named GII.4 2006a and GII.4 2006b in Europe) have caused NoV epidemics since 2006 (Bok et al., 2009; Siebenga et al., 2009; Zheng et al., 2009). Each successive variant has built up a number of mutations in the capsid sequence from its predecessor in time, except for the NoV GII.4 Den Haag variant (Siebenga et al., 2007b). Reasons for this success are thought to be the heavy immune selection of the NoV capsid, leading to an apparent strong antigenic drift to avoid human herd immunity (Lindesmith et al., 2008). Moreover, silent circulation of NoV GII.4 strains in asymptomatic carriers and chronic shredders could allow the accumulation of mutations leading to new strains (Carlsson et al., 2009; Gallimore et al., 2004b; Nilsson et al., 2003). However, both arguments are valid to all NoV genotypes and further research investigating the success of these NoV GII.4 strains is ongoing.

1.2.4. Importance of NoV as a foodborne pathogen

To estimate the importance of NoV as a food borne pathogen compared to other bacterial and viral food borne pathogens, two main sources of aggregated food borne outbreak data are available.

A first source of aggregated NoV food borne outbreak data is available through official bodies such as the Centers for Disease Control and Prevention (CDC, USA), the European Food Safety Authority (EFSA, Europe) or the World Health Organization (WHO). The latter agency considered NoV as member of the Group 1 viruses, with priority in terms of foodborne viral disease. This classification was mainly based on its high incidence (Anonymous, 2008). A second source of aggregated NoV food borne outbreak data are individual initiatives by research groups mostly in Europe and the USA, although limited Asian and Oceanian data are available as well (Table 1.1). While the CDC and EFSA data describe the fraction of all reported food borne outbreaks caused by NoV, most studies on individual initiatives describe the fractions of NoV outbreaks that have a food borne origin.

Food borne outbreaks have been registered and investigated by official bodies in Europe and the USA since several decades and earliest published reports date back to 1969 (Fodor et al., 1970; Gangarosa and Donadio, 1970; Ringertz, 1971; Vernon, 1977). However, main focus of these reports was set to outbreaks caused by bacterial pathogens. Food borne viruses (including noroviruses) have increasingly been included since the discovery and recognition of

the clinical importance of the “Norwalk-like” viral agents (Dolin et al., 1987). Data originating from the EFSA between 2005 and 2008 demonstrate the growing importance of food borne viruses in 24 European countries (Fig. 1.4 and Fig. 1.5; left charts). While these viruses were responsible for 6.5 % (312 out of 4782) for all food borne outbreaks with a known etiology in 2005, this number increased to 12.2 % (593 out of 4855), 16.3 % (682 out of 4176) and 17.6 % (697 out of 3952) in 2006, 2007 and 2008 respectively (Fig 1.4; left chart).

In comparison to other food borne viruses, NoV has been considered the most important food borne viral agent in Europe, as it caused 62.2 % and 61.0 % of all reported viral food borne outbreaks in 2005 and 2006 (Fig 1.5; left chart). This percentage increased to 72.6 % and 79.5 % in 2007 and 2008. In contrast, the fraction of viral food borne outbreaks caused by RoV has decreased from 27.3 % in 2005 and 21.4 % in 2006 to 1.8 % in 2007. In 2008, no food borne RoV outbreaks were reported to EFSA. Finally, the percentage of reported viral food borne outbreaks caused by HAV has also increase from 3.2 % in 2005 to 10.3 % in 2008.

Data collected in the USA by the CDC provided information from 1998 to 2007 (Fig. 1.4 and Fig. 1.5; right charts). Interestingly, the fraction of food borne outbreaks caused by NoV was 2 to 8 fold higher compared to European data. While 28.5 % (346 out of 1212) of reported food borne outbreaks with a known etiology between 1998 and 2000 were caused by food borne viruses, this number increased to 44.5 % (359 out of 806) and even 56.3 % (511 out of 907) in between 2001 and 2006 (Fig 1.4; right chart)(Anonymous, 2009b; Lynch et al., 2006). Although a 12.2 % reduction was noticeable in 2007 compared to 2006, food borne viruses have been responsible for most (44.1 %; 324 out of 734) of the food borne outbreaks with known etiology since 2001 in the USA.

NoV are by far the most important food borne viruses in the USA, as 89.0 % of viral food borne outbreaks between 1998 and 2000 were caused by NoV. This percentage has raised to 98.8 % and 97.8 % in 2006 and 2007, respectively. The second most common food borne virus was hepatitis A virus (Fig 1.5; right chart) (Anonymous, 2009b; Anonymous, 2010a; Lynch et al., 2006).

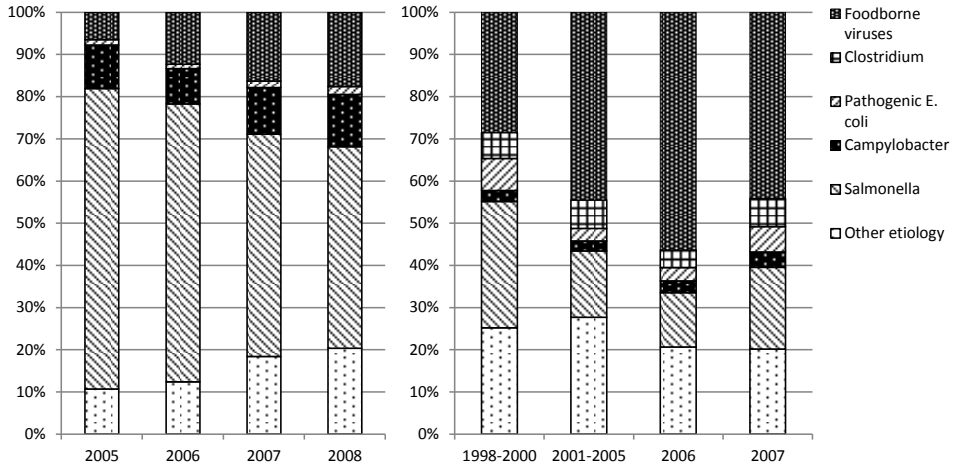

Fig. 1.4 Importance of bacterial pathogens and food borne viruses as cause of food borne outbreaks with confirmed etiology between 2005 and 2008 in Europe (left chart) and between 1998 and 2007 in the USA (right chart) (Anonymous, 2007a; Anonymous, 2007b; Anonymous, 2009a; Anonymous, 2009b; Anonymous, 2010a; Anonymous, 2010b; Lynch et al., 2006).

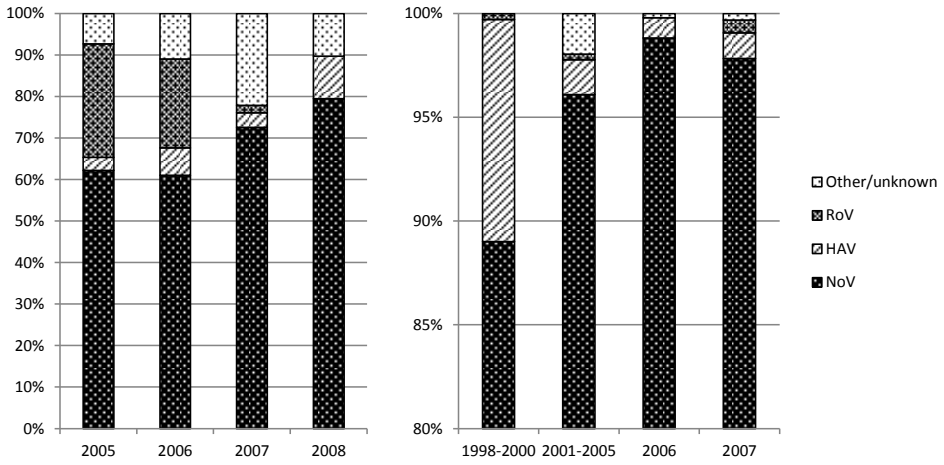

Fig. 1.5 Importance of three food borne viruses (NoV, RoV, HAV) as cause of viral food borne outbreaks between 2005 and 2008 in Europe (left chart) and between 1998 and 2007 in the USA (right chart) (Anonymous, 2007a; Anonymous, 2007b; Anonymous, 2009a; Anonymous, 2009b; Anonymous, 2010a; Anonymous, 2010b; Lynch et al., 2006).

In Europe, two of the most extensive studies on individual initiative by research groups describing NoV food borne outbreaks were performed by the Food borne Viruses in Europe Network (FBVE; http://www.noronet.nl/fbve/). These studies covered 9 and 13 European

countries and indicated that 10.0 % to 17.9 % of all recorded NoV outbreaks between 2001 or 2002 and 2006 had a food borne origin (Kroneman et al., 2008b; Verhoef et al., 2009). Interestingly, except for a German and a Spanish study, studies in single countries confirmed these results as 6.5 % to 14.3 % of described NoV outbreaks showed a food borne cause. The fraction obtained from the Spanish data was 50 %, but could be biased due to the low number of outbreaks investigated (n=60). In Germany, this fraction was only 1.3 %, but this percentage could be explained by the exclusion of non laboratory confirmed outbreaks (Krause et al., 2007; Torner et al., 2008). For the USA, state specific data showed that 22.1 % to 92.9 % of all reported NoV outbreaks had a food borne origin (Doyle et al., 2009; Jenkins et al., 2007; Tseng et al., 2007). However, these percentages could be biased due to the low number of outbreaks included in these studies. Similar to the USA data, most studies describing food borne NoV outbreaks in Japan were compiled per province (Hamano et al., 2005a; Iijima et al., 2008) and these studies pointed out that 6.3 % to 34.7 % of recorded NoV outbreaks were food borne. Finally, three studies described NoV outbreaks in Australia and New-Zeeland, reporting only a very limited number (8 to 46) of food borne NoV outbreaks (Table 1.1)(Bruggink and Marshall, 2010; Marshall et al., 2005; Widdowson et al., 2005).

Table 1.1 Overview of data on NoV food borne outbreaks collected by research groups on own initiatives.

Continent	Country	Period	NoV OB[a]	NoV FBO[b]	NoV FBO / NoV OB	Ref
Europe	England and Wales	1992 – 2000	1877	184	9,8%	(Lopman et al., 2003a)
	England and Wales	1992 – 1995	707	96	13,6%	(Dedman et al., 1998)
	United Kingdom	1996 – 1997	64	7	10,9%	(Maguire et al., 1999)
	The Netherlands	1994 – 2005	735	48	6,5%	(Svraka et al., 2007)
	The Netherlands	1994 – 2005	695	46	6,6%	(Siebenga et al., 2007a)
	The Netherlands	2002	151	15	9,9%	(van Duynhoven et al., 2005)
	Spain (Catalonia)	2004 – 2005	60	30	50,0%	(Torner et al., 2008)
	Norway	2000 – 2005	204	29	14,2%	(Vainio and Myrmel, 2006)
	Finland	1998 – 2002	252	36	14,3%	(Maunula and von Bonsdorff, 2005)
	Germany[c]	2004 – 2005	1239	16	1,3%	(Krause et al., 2007)
	13 countries	2001 – 2006	5036	506	10,0%	(Kroneman et al., 2008b)
	9 countries	2002 – 2006	1254	224	17,9%	(Verhoef et al., 2009)
North America	USA (North Carolina)	2006	17	4	23,5%	(Jenkins et al., 2007)
	USA (North Carolina)	1995 – 2000	14	13	92,9%	(Tseng et al., 2007)
	USA (Florida)	2006 – 2007	113	25	22,1%	(Doyle et al., 2009)
Asia	Japan (Okayama)	1997 – 2004	46	16	34,8%	(Hamano et al., 2005b)
	Japan (Kobe)	2006	95	6	6,3%	(Iijima et al., 2008)
	China (Hong Kong)	2001 – 2002	44	18	40,9%	(Lau et al., 2004)
Oceania	Australia (Victoria)	2001	30	8	26,7%	(Marshall et al., 2005)

[a] NoV OB: NoV outbreaks, [b] NoV FBO: NoV related food borne outbreaks, [c] only verified outbreaks included

1.2.5. Transmission routes of NoV

Faecal-oral spread is generally the most important mode of transmission for NoV, although various transmission routes exist. A schematic overview of proven and hypothetical transmission routes is shown in Fig 1.6. The spread of NoV by transmission routes related to foods, water and person-to-person contact is facilitated by a number of factors such as (1) the low infectious dose of 10 to 100 infectious virus particles (Teunis et al., 2008), (2) prolonged duration of viral shedding, even after resolving of the symptoms, (3) asymptomatic NoV infections, (4) the stability of the virus in relatively high concentrations of chlorine and at a wide range of temperatures (from freezing to 60°C) and (5) the lack of complete cross-protection against the diverse NoV strains and an inadequate long-term immunity.

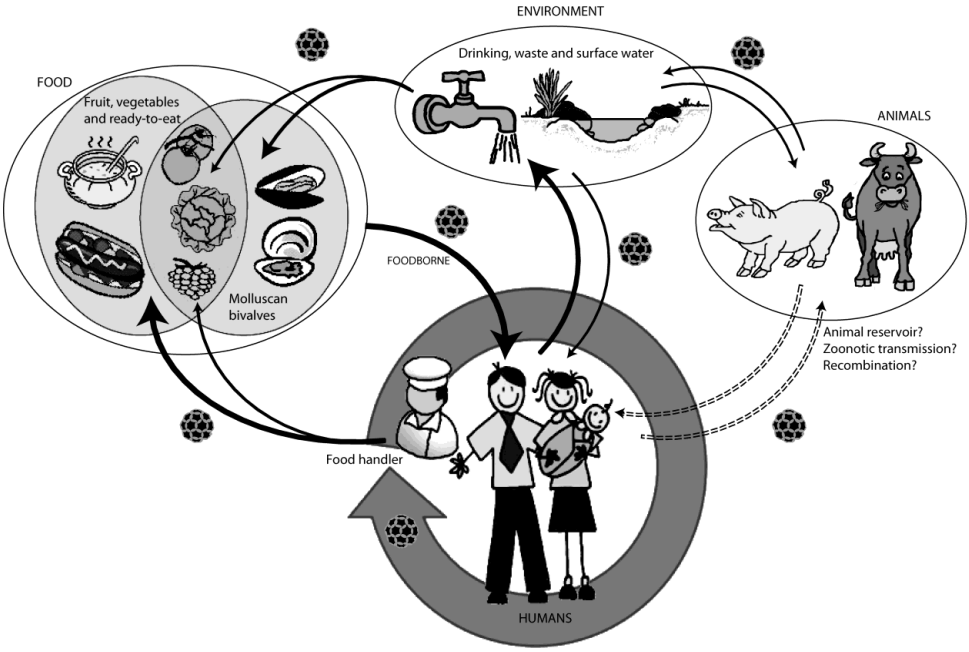

Fig. 1.6 Schematic overview of the transmission routes of NoV. Solid and dashed arrows indicate proven and hypothetical transmission routes, respectively. The thickness of the arrows is related to the likeliness of the transmission route.

Transmission routes not related to consumption of contaminated foods include person-to-person transmission and water borne transmission. Although NoV can be transmitted through several routes, person-to-person transmission is considered to be most important. Primary cases in NoV outbreaks often have a food or water borne cause, whereas person-to-person spread among contacts of primary cases may further propagate the epidemic. NoV

outbreaks related to this transmission are extensively spread in semi-closed community settings such as hospitals, cruise ships, day-care centers and military settings and can implicate a few to a lot of people (Gallimore et al., 2004a; Gallimore et al., 2004b; Grotto et al., 2004; Takkinen, 2006). On the other hand, a large number of people, up to several thousands, are generally involved in water borne outbreaks. Water borne outbreaks can be related to different water types such as distribution water, borehole water, well water, flood water and water used for recreational purposes. The hypothesis of a zoonotic reservoir for human NoV has been investigated by several research groups, but no proof has been provided for its existence to date. Nevertheless, a phenomenon that could lead to zoonotic NoV is co-infection of humans or animals, followed by recombination of human and animal NoV (Bank-Wolf et al., 2010). Co-infection of oysters with human and porcine NoV has been observed, and recombination between NoV (human or animal) has been known to occur (Bull et al., 2007; Mathijs et al., 2010). However, a recent qualitative risk assessment performed to investigate the zoonotic potential of animal diseases categorized bovine NoV in level 0, with level 0: not zoonotic to level 4: confirmed zoonosis (Palmer et al., 2005).

To investigate the transmission routes related to consumption of NoV contaminated foods, a literature review of individual NoV food borne outbreaks occurring between 2000 and 2010 was performed, covering 51 studies and 59 outbreaks. However, the number of described outbreaks is considered as a serious underestimation of the actual number and is probably biased because peer-reviewed publications of food borne outbreaks report merely large, well-documented, unusual or novel events (Baert et al., 2009b; Kroneman et al., 2008a; O'Brien et al., 2006).

The reviewed NoV food borne outbreaks data were compiled in Table 1.2 and categorized per transmission route: by pre-harvest contamination or via infected food handlers, either confirmed or suspected. The involvement of an infected food handler was considered confirmed if (1) epidemiological analysis showed that the food handler was infected before the food borne outbreak and could be linked to manipulation of the involved food products and if (2) laboratory analysis showed identical NoV genogroups or genotypes in the clinical samples from both patients and food handler. For every NoV food borne outbreak in Table 1.2, the attack rate and/or the number of affected people and the laboratory confirmation of NoV presence in human and/or food samples was described. Overall, reverse transcriptase polymerase chain reaction (RT-PCR), whether or not in real-time format, was the most used molecular detection method, occasionally in combination with an enzyme immuno assay (EIA) or electron microscopy (EM). Extraction of virus particles or viral genomic material from the food samples was performed by various methods, although frequently not specified.

1.2.5.1. Pre-harvest contamination of produce and bivalve shellfish by water.

NoV outbreaks (n = 24 out of 59) caused by pre-harvest contamination were related to consumption of produce and shellfish whereby mainly raspberries (42 %) and oysters (33 %) were involved in these outbreaks (Table 1.2). An average attack rate of 51 % was observed with on average 145 people involved per outbreak. The most observed NoV genotype in clinical and food samples was GII.4, although NoV genotypes GI.1, GI.2 , GI.4 and GII.7 were frequently detected in food and clinical samples as well. In total, 13 different NoV genotypes were detected. In 42 % (10 out of 24) of these outbreaks, identical genogroups or genotypes were found in clinical and food samples. In an additional 8.3 % (2 out of 24) of these NoV food borne outbreaks, different NoV genotypes were detected in clinical and food samples. Noteworthy, NoV could be detected in 82 % (9 out of 11) of shellfish samples, which may be explained by the higher levels of NoV found in shellfish compared to other food products due to their filter feeding capability. This concentration of higher NoV levels (in the digestive tissue) facilitated development of enteric virus detection methods in this matrix. Therefore, the prevalence of food borne viruses (and in particular NoV) in shellfish and in harvesting water has been documented more extensively compared to other food products. A three year survey between 2005 and 2008 investigating 116 retail shellfish samples (mussels, clams and oysters) showed a confirmed (sequenced) detection of GII NoV (genotypes GII.4 2004 and GIIb) in 10.3 % of all tested samples (Terio et al., 2010). Likewise, GII.4 and GIIb genotypes were found in 16.7 % of oyster and mussel samples (n = 42) during a 2 year survey for NoV prevalence in Dutch shellfish (Boxman et al., 2006) and a 1 year survey in 235 Italian shellfish samples showed presence of NoV and HAV in 13.2 % and 2.2 % of all tested samples, respectively (Croci et al., 2007). A lower NoV prevalence was observed when 1512 Japanese oysters were screened for NoV prevalence as a broad range of GI and GII NoV genotypes were found in 4.9 % of all tested oyster samples (Nishida et al., 2007). NoV have been detected in sewage with high concentrations (339 to 10^6 NoV genomic copies per liter) and treatment of the sewage caused a minor reduction of 0.7 to 2.7 logs NoV genomic copies per liter (Lodder and de Roda Husman, 2005; van den Berg et al., 2005). A Japanese study has shown that very similar NoV genotypes can be detected in human feces, domestic sewage, treated wastewater, river water and in cultivated oysters (Ueki et al., 2005), demonstrating that transmission of NoV from contaminated harvesting water to bivalve filterfeeding shellfish such as mussels or oysters is possible. Due to the more difficult detection of enteric viruses in produce compared to shellfish, only a limited number of studies has investigated the prevalence of enteric viruses on this food matrix. In a recent study by Mattison et al. (2010b) NoV genomic material was detected in 148 out of 275 tested packaged leafy greens by real-time RT-PCR, but only 16 samples could be confirmed by sequencing. In total, 13 samples tested positive for NoV GI genotypes (GI.2/3/4/6/8) and 3

samples for NoV GII (all GII.4). NoV presence (together with AdV) has also been detected in a single spinach sample when screening 30 produce samples for enteric virus presence (Cheong et al., 2009b). NoV have also been detected in river water, in general at lower concentrations compared to treated and untreated sewage (1.6 to 2 × 10^3 NoV genomic copies per liter) (Laverick et al., 2004; Lodder and de Roda Husman, 2005). Although no studies have investigated the presence of NoV in irrigation water, presence of identical RoV has been shown in irrigation water and in a related tomato sample (van Zyl et al., 2006).

1.2.5.2. Contamination of food by the food handler.

Table 1.2 shows that food handlers were involved in 35 out of 59 NoV food borne outbreaks, either suspected (46 %) or confirmed (54 %). Deli sandwiches were the mostly implicated foods in these food borne outbreaks, together with a wide range of food products such as various catered meals, buffet foods, fresh produce and shellfish. An average attack rate of 34 % was observed with 120 people averagely involved per outbreak. Detection of NoV in foods related to these food borne outbreaks associated with an infected food handler was more difficult in contrast to outbreaks caused by pre-harvest contamination of produce and shellfish as NoV could only be detected in 11 % (4 out of 35) of these NoV food borne outbreaks. The most frequently detected NoV genotype in clinical samples was the GII.4 genotype, although NoV GI.3 was detected in some outbreaks as well.

The role of the food handler is considered an important route for transmission of food borne viruses, as several factors can contribute to this transmission. Firstly, food handlers carrying an asymptomatic NoV infection can easily cause food borne outbreaks since these people can shed similarly high NoV levels (Ozawa et al., 2007). Asymptomatic infection of a food handler has been related to NoV food borne outbreaks caused by consumption of deli sandwiches, prepared meals and salad vegetables (Godoy et al., 2005; Ohwaki et al., 2009; Vivancos et al., 2009). In some cases, poor personal hygiene has been reported as well (Rizzo et al., 2007; Schmid et al., 2007b), often in combination with ill food handlers still coming to work (Grotto et al., 2004). In addition, lack of respect to hygienic working circumstances can contribute to NoV food borne outbreaks (Friedman et al., 2005; Schmid et al., 2007b). For example, sinks used for both washing hands and washing lettuce have been related to a NoV food borne outbreak (Payne et al., 2006). Another study reported vomiting of an ill baker in a sink in the food preparation area which resulted in NoV contamination of deli sandwiches (De Wit et al., 2007). Since food handlers are often related to large catering establishments, outbreaks related to this transmission route tend to affect a lot of people at once (Noda et al., 2008). Therefore, an apparent infection should always be reported to avoid these outbreaks and an infected food handler should not be at a work place where foods are manipulated (Vivancos et al., 2009).

Table 1.2 Overview of NoV food- and water borne outbreak data between 2000 and 2010

	Foods involved	Attack rate (%)	Laboratory investigations		Detection methodology		Reference
			Human samples	NoV in Food/food handler samples	Virus extraction (food)	Molecular detection method (food + clinical)	
Pre-harvest contamination	Raspberries (in preparations)	30 people	5/9 stool: GI	Raspberries: GIIb	Alkaline elution - PEG concentration	RT-PCR	(Le Guyader et al., 2004a)
	Raspberries (frozen)	1043 people (6 outbreaks)	Stool: GII.7, GII.4, GIIb			RT-PCR	(Falkenhorst et al., 2005)
	Raspberries (frozen)	74/270 (**27 %**)	5/6 stool: GI.5			ns	(Cotterelle et al., 2009)
	Raspberries (in preparations)	43/74 (**58 %**)	5/5 stool: NoV			RT-PCR	(Hjertqvist et al., 2006)
	Raspberries	~200 person s	2/2 stool: GI.4	3/5 raspberries: GI.4	Ns[a]	RT-PCR	(Maunula et al., 2009)
	Salad vegetables	>400 people	11/11 stool: GII.I		Alkaline elution - ultrafiltration	Real-time RT-PCR	(Makary et al., 2009)
	Salad vegetables	23 people	25/26 stool: GII.4	1 salad vegetable: GII	ns	Real-time RT-PCR	(Oogane et al., 2008)
	Lettuce	260/480 (**54 %**)	2/25: GI, 12/25: GII, GI+GII: 9/25	1/2 lettuce heads: GII	ns	ns	(Ethelberg et al., 2010)
	Oysters	30/100 (**30 %**)	1/1 stool: GI.I	3/5 oysters: GI.I, GII.3	Direct RNA extraction	Real-time RT-PCR	(Nenonen et al., 2009)
	Oysters	83/106 (**78 %**)	8/8 stool: GI.2/4,GII.5/6/7/9/12	Oysters: GII.4	Proteinase K treatment	RT-PCR	(Webby et al., 2007)
	Oysters	205 people	9/12 stool: GI.I/2,GII.4/7	62 Oysters: 25 GI, 20 GII.4	Proteinase K treatment	Real-time RT-PCR	(Le Guyader et al., 2008)
	Oysters	14 cases	2/4 stool: GI.I	5/6 oysters: GI.I	Proteinase K treatment	RT-PCR	(Le Guyader et al., 2003)
	Oysters	15/22 (**68 %**)	11/11 stool: GI and GII			(hemi-nested) RT-PCR	(Gallimore et al. 2005)
	Oysters	53 people	26/53 stool: GI.2	Oysters: GI.2	Neutral elution - PEG concentration	RT-PCR	(David et al., 2007)
	Oysters (French)	202 people	29/53 stool: GI.4/6,GII.4/8/b	3/3 shellfish: GI.4, GII.4, GII.8	Proteinase K treatment	RT-PCR	(Le Guyader et al., 2006)
	Oysters (frozen half shelled)	305 people	4/5 stool: GII	6/11 oysters: GII	ns	Real-time RT-PCR	(Ng et al., 2005)
	Mussels	103/139 (**74 %**)	24/24 stool: GI and GII	6/11 mussels: GI and GII	ns	Real-time RT-PCR	(Prato et al., 2004)
	Mussels (raw) / ice	~400 people	18/20 stool: GII			RT-PCR	(Rizzo et al., 2007)
	Clam s	5 people		59 pooled clams: GII	Alkaline elution - PEG concentration	RT-PCR	(Kingsley et al., 2002)
Food handler involvement confirmed	Deli sandwich	140/231 (**61 %**)	15/16 stool: NoV	5/8 food handlers: NoV		RT-PCR	(de Coster et al., 2001)
	Deli sandwich	38/57 (**67 %**)	12/14 stool: NoV	2/4 food handlers: NoV		RT-PCR	(Godoy et al., 2005)
	Deli Sandwiches	231/505 (**46 %**)	24/27 stool: GII	Baker: GII		RT-PCR	(De Wit et al., 2007)
	Deli sandwich/salad	34/427 (**1 %**)	12/14 stool: GI.3 Desert Shield	Cook: GI.3		RT-PCR	(Sala et al., 2005)
	Deli sandwiches	87/142 (**61 %**)	21/21 stool: NoV	Food handler: NoV		RT-PCR	(Payne et al., 2006)
	Catered meal (hospital)	102/698 (**15 %**)	23/32 stool: GII.4	15/23 Food handler: NoV		Real-time RT-PCR	(Ohwaki et al., 2009)
	Restaurant lunch	660/1492 (**44 %**)	87/124 stool and vomit: GII.2	5/10 food handlers: GII.2		Real-time RT-PCR	(Hirakata et al., 2005)
	Restaurant lunch	364/584 (**62 %**)	14/14 stool: GI.4 Chiba	Food handler: NoV		RT-PCR	(Bohm et al., 2008)
	Catered meal (canteen)	120/1357 (**9 %**)	19/19 stool: NoV	Food handler + cook: NoV		RT-PCR	(Lederer et al., 2005)
	Mixed salad	36 people	3/9 stool: GII.4	Food handler: GII.4		ns	(Showell et al., 2007)
	Salads	333/753 (**44 %**)	32/59 stool: GII	2/15 food handlers: NoV ab[b]	Direct RNA extraction	RT-PCR	(Anderson et al., 2001)

	Salad vegetables	182/325 (**56 %**)	5/6 stool: GII.7 Leeds	4/5 kitchen staff: GII.7		Real-time RT-PCR	(Schmid et al., 2007a)
	Salad vegetables	60/106 (**57 %**)	6/13 stool: GII.6 Seacroft	Food handler:GII.6		Real-time RT-PCR + EIA[c]	(Vivancos et al., 2009)
	Salad buffet vegetables	413/1744 (**24 %**)	8/28: GI.3 Desert Shield	Food handler: GI.3		RT-PCR	(Zomer et al., 2009)
	Canteen food (salads)	101/815 (**12 %**)	12/53 stool: GII.4 2006b	4/25 canteen workers: GII.4 2006b	Elution-precipitation/ultracentrifugation		(Wadl et al., 2010)
	Soup	35/61 (**57 %**)	24/31: GII.4 Terneuzen	5 food handlers: GII.4 Terneuzen		RT-PCR + EM	(Medici et al., 2008)
	Ham rolls	21/63 (**33 %**)	3/21: GII	Kitchen assistant: GII		RT-PCR	(Kuo et al., 2009)
	Wedding cake	332/805 (**41 %**)	4/4 stool: NoV	Food handler: NoV		RT-PCR	(Friedman et al., 2005)
	Shellfish	3/4 (**75 %**)	2/4 stool: NoV	Food handler: NoV		RT-PCR	(Sala et al., 2009)
Food handler involvement suspected	Catered meal (retirement home)	45/83 (**54 %**)	31/36 stool: NoV			RT-PCR	(Kiehl et al., 2001)
	Catered meal (school excursion)	63/85 (**74 %**)	5/5 stool: GII			RT-PCR	(Parasidis et al., 2007)
	Prepared meal (private home)	28/48 (**58 %**)	12/13 stool: GII.4			RT-PCR	(Glasscock et al., 2007)
	Restaurant lunch	158/219 (**72 %**)	7/8 stool: GI			RT-PCR + EM	(Johansson et al., 2002)
	Banquet	33/83 (**40 %**)	4/4 stool: GI.3			RT-PCR	(Nordgren et al., 2010b)
	Meal (ns)	21/25 (**84 %**)	2/2 stool: GI			RT-PCR	(Fretz et al., 2005)
	Buffet food (catered event)	>40 people	4/4 stool samples: GIIb	Ham, salami: GIIb	Direct RNA extraction	Semi-nested RT-PCR	(Boxman et al., 2007)
	Buffet food (private home)	12/21 (**57 %**)	no stool samples	Ham off the bone: GII.4 2004	Direct RNA extraction	Semi-nested RT-PCR	(Boxman et al., 2007)
	School lunch	85/106 (**80 %**)	4/6 stool: GII			Real-time RT-PCR	(Morioka et al., 2006)
	Take-out food	6/6 (**100 %**)	no stool samples	Sparerib: GIIb	Direct RNA extraction	Semi-nested RT-PCR	(Boxman et al., 2007)
	Rice cocktail	38 people	2/4 stool: NoV			ns	(Costas et al., 2007)
	Lunchboxes	99 people	21/23 stool: GI.12			RT-PCR	(Sakon et al., 2005)
	Salad	64/113 (**57 %**)	3/3 stool: GI			RT-PCR	(Dippold et al., 2003)
	Canteen food (salad)	159 people	4/24 stool: GII			RT-PCR	(Grotto et al., 2004)
	Lettuce salad and soup	16/22 (**73 %**)	2/2 stool, 1/1 emesis: GII.4 2006b			RT-PCR	(Mesquita and Nascimento, 2009)
	Raspberries + water	48/137 (**35 %**)	6/17 stool: GII.4 2006b	Raspberries: GII	Direct RNA extraction (food swabs)	RT-PCR	(Verhoef et al., 2008)

[a] ns: not specified, [b] ab: antibodies, [c] EIA: enzyme immuno assay

1.3. Detection of food borne viruses

1.3.1. Clinical vs food samples

Food samples (together with environmental samples) on one hand and clinical samples on the other hand are two categories of samples that differ in some crucial aspects.

Firstly, clinical samples (faeces and emesis) can contain up to 10^{10} NoV genomic copies/g (Atmar et al., 2008; Lee et al., 2007), while food samples generally contain much lower viral concentrations such as 10^2 to 10^4 NoV genomic copies per gram digestive tissue in naturally contaminated shellfish samples (Le Guyader et al., 2006; Le Guyader et al., 2009; Nishida et al., 2007).

Secondly, while both clinical and food samples can contain inhibitory substances interfering with molecular detection (Escobar-Herrera et al., 2006; Rijpens and Herman, 2002), this is especially a problem regarding the investigation of NoV presence in the latter class of samples due to the mentioned low viral concentration. Common food components such as polysaccharides, proteins and fat molecules are known to inhibit molecular assays like RT-PCR (Demeke and Jenkins, 2010; Schwab and McDevitt, 2003).

Therefore, the strategies for detection of NoV in clinical and food samples are different. While the RNA extraction/purification (paragraph 1.3.5) and molecular detection (paragraph 1.3.6) steps might be similar for both sample categories, food samples require preceding steps for (1) virus extraction and concentration and (2) inhibitor removal from the sample matrix (paragraph 1.3.3 and 1.3.4).

1.3.2. NoV detection strategy

In contrast to food borne bacteria, detection of NoV in food samples requires a virus extraction step since enrichment is not possible (Duizer et al., 2004). Currently, the four main used virus extraction approaches are based on the principles of (1) elution – concentration, (2) acid adsorption – elution – concentration, (3) direct RNA extraction or (4) proteinase K treatment. Depending on the food type, one or multiple approaches are suitable (Fig. 1.6).

Subsequently, an RNA purification step is required. In case an (acid adsorption –) elution – concentration or proteinase K based approach is used, RNA purification is needed to extract the viral RNA from its capsid as well as to remove possible inhibitory substances. On the other hand, after direct RNA extraction, RNA purification is mainly used for the latter purpose. Most common RNA purification methods are based either on the use of guanidine isothiocyanate (GITC) and phenol or on the use of nucleic acid binding silica beads.

Finally, the purified RNA is detected by a molecular assay. Reverse transcriptase polymerase chain reaction (RT-PCR) and nucleic acid sequence based amplification (NASBA) are the most common used assays for detection of NoV RNA. Both methods can be used in a real-time format. If a molecular method is included, the detection limits of the

NoV detection methods are described either as RNA copies or RT-PCR units (RT-PCRU) per analyzed mass (in gram). An RT-PCRU is the lowest amount of viral genomic material that can be detected when using RT-PCR for detection of NoV and can thus differ between different assays.

The use of appropriate positive controls throughout the different steps of the NoV detection protocols is required to avoid detection of false negative results caused by reaction inhibition due to malfunction of thermal cycler, incorrect PCR mixture, poor DNA polymerase activity, incorrect execution of the virus detection protocol, or not least the presence of inhibitory substances in the sample matrix (Hoorfar et al., 2004). On the other hand, since false-positive results are often caused by cross-contamination (Rijpens and Herman, 2002), the inclusion of negative controls in the detection methodology is important to detect this contamination.

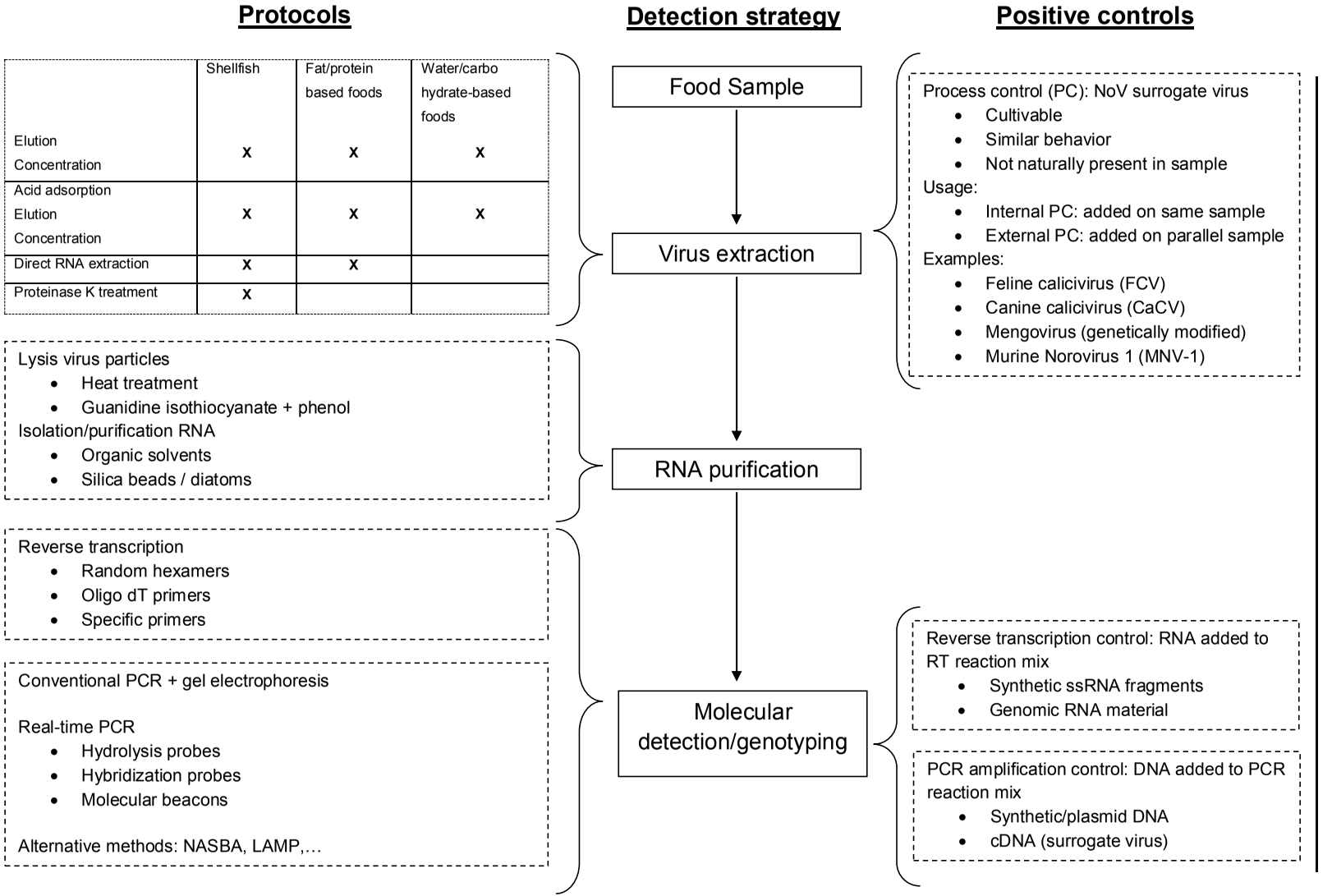

Fig. 1.6 overview of strategies for detection of viral agents in food products, including appropriate protocols and controls for the virus extraction step, RNA purification and molecular detection/genotyping steps.

1.3.3. Virus extraction

The virus extraction method used when detecting viral agents in food products is dependent on the food type composition. As proposed by Baert and colleagues (2008a), two main food type categories are distinguished besides shellfish which forms an apart food category (Fig. 1.7).

The first category of foods is considered as “carbohydrate/water based foods” and includes mostly fresh produce (fruits and vegetables). These foods consist mainly of carbohydrates and water, while the second category of foods are mainly composed of fat and proteins. The latter category of foods exists mostly of “ready-to-eat foods” (such as deli food products). Shellfish are considered as a special food category due to their ability to filter large volumes of water as part of their feeding activities, enabling the accumulation and concentration of viral particles in the digestive glands (Le Guyader et al., 2009). Therefore, separate viral extraction protocols have been developed for this food category.

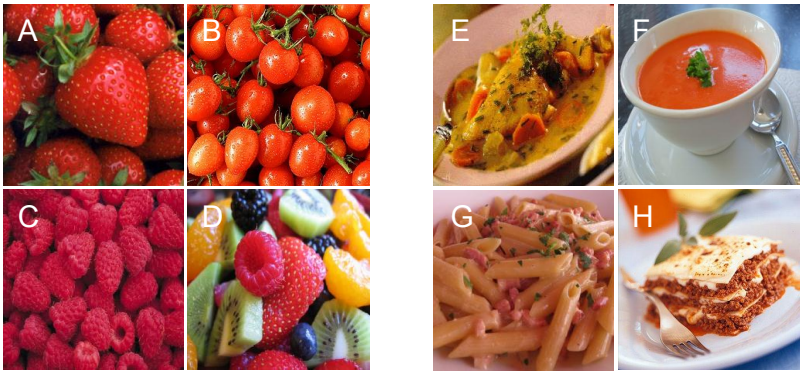

Fig. 1.7 Examples of carbohydrate/water based foods (left side figures A - D) and fat/protein based foods (right side figures E - H)

A first issue regarding these virus extraction methods is that although a large number of methods have been proposed for all three food types, no standardized method has been approved yet. Therefore, most labs are restricted to their in-house developed methods for these food types. A possible reason for this lack of standardization is the limited attention spent on the evaluation and validation of developed elution-concentration methods. However, recent efforts have been made within various EU projects, between reference laboratories and within the European Committee for Standardization/Technical Committee 275/Working Group 6/Task Group 4 on virus detection in foods (CEN/TC275/WG6/TAG4) to stimulate the acceptance of standardized methods for detection of most common enteropathogenic viruses in produce, shellfish and bottled water (Croci et al., 2008; Rodriguez-Lazaro et al., 2007). A second issue is the high variability and variance in the recovery (both quantitative and qualitative) of viruses from food products. Although this might

be intrinsic to the used methods since a large number of steps is involved in most procedures, a possible solution might be the use of an adequate (quantifiable) process control.

Virus extraction of the three food categories (carbohydrate/water based foods, fat/protein based foods, shellfish) is performed using a variety of described protocols, although four main approaches are most often used. These approaches are (1) elution of the virus particles from the food surface followed by concentration of the eluted virus particles, (2) acid adsorption of the virus particles on the food surface, followed by elution and concentration of the eluted virus particles, (3) direct extraction of the viral RNA from the food matrix without preceding elution/concentration step, and finally (4) extraction of the virus particles from the food by proteinase K treatment (Fig. 1.6).

Most virus extraction methods for carbohydrate/water based foods (typically fruits and vegetables) are based on the elution-concentration approach, whether or not in combination with an acid adsorption step. While the elution step is quite similar in most protocols, several concentration methods are used such as precipitation by polyethylene glycol (PEG), ultracentrifugation and ultrafiltration. A single study has described a direct RNA extraction protocol for detection of HAV from cilantro leaves (Goswami et al., 2002). Virus particles are extracted from fat/protein based foods using either the acid adsorption – elution – (immunomagnetic) concentration (Love et al., 2008; Morton et al., 2009) or the elution – concentration approach (Fumian et al., 2009; Kobayashi et al., 2004; Leggitt and Jaykus, 2000; Papafragkou et al., 2008; Rutjes et al., 2006a; Scherer et al., 2010). Direct RNA extraction has been reported as well (Baert et al., 2008a; Boxman et al., 2007; Kim et al., 2008b; Schwab et al., 2000). Finally, several approaches have been described for virus extraction in shellfish. Beside the mentioned approaches for carbohydrate/water based foods and fat/protein based foods, an approach using a proteinase K treatment has successfully been tested for shellfish (Comelli et al., 2008; Jothikumar et al., 2005b). Virus extraction can either be performed from whole shellfish tissue or from the digestive tissue isolated from the shellfish. The latter approach can be useful since shellfish are filter-feeders, resulting in the accumulation of viral pathogens in the digestive tissue (Bosch, 2010).

1.3.3.1. Elution – concentration protocols

Elution – concentration protocols have been used for all food types, although the different food matrices require adjustments towards the used protocol, in particular regarding the purification of the virus eluate and the virus concentrate. The principle is based on elution of the virus particles from the food surface using an appropriate buffer, followed by concentration of the eluted viruses using a variety of methods. Results obtained using different elution-concentration protocols are summarized in Table 1.3.

Virus particle elution

In most cases, an alkaline buffer (pH between 9 and 10.5) has been used to elute the viral agents from the food surface. These alkalic pH levels allowed detachment of the virus particles from the food surface, while acidic pH levels allow attachment of virus particles on the foods, thereby impairing virus elution and consequently reducing detection sensitivity (Dubois et al., 2002; Traore et al., 1998). The latter principle is used for acid adsorption of virus particles on the food surface. Noteworthy, when anionic exchange or ultracentrifugation is subsequently used for concentration of the eluted virus particles, a neutral buffer is used (Fumian et al., 2009; Morales-Rayas et al., 2009; Morales-Rayas et al., 2010; Rutjes et al., 2006a; Rzezutka et al., 2008).

Since many food products contain acidic substances (in particular fruits and vegetables which contain acidic fluids), a buffer system is required to prevent pH drops when extracting viruses and usually an alkaline Tris-based buffer system is applied for this purpose (Cheong et al., 2009a; Scherer et al., 2010; Sincero et al., 2006). However, other elution buffers have been described. Firstly, a phosphate buffer has been used (pH 7.6) to elute HAV from lettuce and fresh strawberries (Bidawid et al., 2000), NoV from rolled cabbage and macaroni (Kobayashi et al., 2004), canine calicicvirus (CaCV) from whipped cream (Rutjes et al., 2006a) and HAV/NoV from oysters (Le Guyader et al., 1998). Secondly, a 1M sodium bicarbonate buffer for recovery of poliovirus from soft fruits and salad vegetables (Kurdziel et al., 2001) has been used.

Table 1.3 Overview of observed detection limits and recoveries per elution-concentration approach and per food matrix. It should be noted that direct comparison of observed detection limits and recoveries is not possible, since the sensitivity of the used RNA purification method and molecular detection method can influence these values.

Protocol						
Elution	**Concentration**	**Food matrix (weight)**	**Viral agent**	**Detection limit (by (real-time) RT-PCR)**	**Recovery**	**Reference**
Alkaline buffer	PEG	Mixed lettuce, fruit salad, raspberries (10g)	NoV GII	10^2 RT-PCRU/10g		(Baert et al., 2008a)
		Strawberries, lettuce (25-100g)	NoV	48 RT-PCRU	2.9 % to 3.4 %	(Cheong et al., 2009a)
		Berries & vegetables (30-100g)	HAV PV NoV	1.2×10^2 $TCID_{50}$ 10^2 $TCID_{50}$ 1.5×10^2 RT-PCRU	17 % PV: 45 % NoV13 %	(Dubois et al., 2002)
		Green onions (25g)	HAV NoV GII	10^2 $TCID_{50}$ 1 RT-PCRU		(Guevremont et al., 2006)
		Strawberries (20g)	NoV GI/GII		NoV GI: 29.5 % NoV GII: 14.14 %	(Park et al., 2008)
		Grapes, strawberries, raspberries (20g)	NoV		12.4 %	(Kim et al., 2008a)
		Tomato sauce, strawberries (30g)	HAV MS2	14 PFU 33 PFU		(Love et al., 2008)
		Lettuce (50g)	NoV	1.5×10^3 RT-PCRU		(Leggitt and Jaykus, 2000)
		Lettuce (8g)	NoV	10 RT-PCRU		(Le Guyader et al., 2004b)
		Oysters, clams (25 g)	NoV HAV	22.4 $RT\text{-}PCRU_{50}$ [a] 0.015 PFU		(Kingsley and Richards, 2001)
		Mussels (2 g)	NoV GII	20-100 RT-PCRU		(Baert et al., 2007)
		Mussels (2 g)	HAV	10 RT-PCRU		(Sincero et al., 2006)
		Whipped cream (5 g)	CaCV		10 %	(Rutjes et al., 2006a)
		Ham (10 g)	NoV GII		10 %	
		Hamburger (50 g)	NoV HAV	1.5×10^3 RT-PCRU 9×10^2 RT-PCRU		(Leggitt and Jaykus, 2000)
Alkaline buffer	Ultrafiltration	Various berries/vegetables (15g)	NoV HAV RoV	54 RT-PCRU 1.2 $TCID_{50}$ 0.02 $TCID_{50}$		(Butot et al., 2010)
		Ham (10 g)	NoV GII	2×10^2 RT-PCRU	10 %	(Scherer et al., 2010)
Neutral buffer	Ultracentrifugation	Raspberries, strawberries (60g)	NoV GI HAV	 10^3 RT-PCRU	NoV GI: 10 %	(Rzezutka et al., 2005; Rzezutka et al., 2006)
		Lettuce (5g)	CaCV		10 %	(Rutjes et al., 2006a)
		Oysters (25 g)	HAV		9.9 %	(Casas et al., 2007)
		Roast pork chop, salami, gammon (20 g)	FCV		3.4 – 12.5 %	(Rzezutka et al., 2008)

		Lettuce, strawberries, green onions (25g)	HAV	$2 \times 10 – 10^5$ RT-PCRU		(Papafragkou et al., 2008)
		Oysters (5 g)	HAV	$2.5 \times 10 – 10^2$ RT-PCRU		(Papafragkou et al., 2008)
		Deli turkey (25 g)	HAV	2.5×10^2 RT-PCRU		
		Cake (25 g)	HAV	2.5×10^5 RT-PCRU		
Alkaline/neutral buffer	Cationic separation	Lettuce, strawberries, green onions, raspberries (50g)	NoV GII HAV MNV-1	10^2 RT-PCRU 10^2 RT-PCRU 10 PFU	NoV: 5 – 16 % HAV: 12 – 33 %	(Morales-Rayas et al., 2009)
		Lettuce (15g)	NoV GII	9.5×10^3 RNA copies	5.2 – 72.3 %	(Fumian et al., 2009)
		Minas Cheese (15 g)	NoV GII	4.1×10^4 RNA copies	6.0 – 56.3 %	(Fumian et al., 2009)
		Lettuce, green onions, strawberries (25g)	NoV	10 RNA copies		(Morton et al., 2009)

[a] Concentration whereby 50 % of inoculations could be recovered.

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Components frequently used in combination with the (alkaline) elution buffers when extracting viruses from various foods are beef extract and glycine, both having the capability to reduce non-specific adsorption of the virus to the food matrix during virus extraction (Dubois et al., 2002). The high protein concentration of beef extracts furthermore facilitates flocculation of NoV on polyethylene glycol molecules (Kim et al., 2008a) and concentrations of 1 % (Blaise-Boisseau et al., 2010; Dubois et al., 2007) and 3 % have been described (de Paula et al., 2010; Love et al., 2008). The use of 1 % (w/v) beef extract has resulted in a 7 fold, 3.5 fold and 5.7 fold increase in recovery of respectively HAV, NoV and RoV from raspberries, combined with the use of an alkaline elution buffer (Butot et al., 2007). However, beef extract might also interfere with molecular detection methods, possibly resulting in false negative results (Katayama et al., 2002; Schwab et al., 2000; Schwab and McDevitt, 2003; Traore et al., 1998). Therefore, a virus elute purification step can be required when using beef extract in the elution buffer. Regarding the use of glycine, a 0.05 M concentration is most often used (Baert et al., 2008a; Cheong et al., 2009a; Scherer et al., 2010). Another component frequently added to the elution buffers when extracting viruses from carbohydrate/water based foods is pectinase, which prevents jelly formation in the eluate by breaking pectin bonds in fruits and vegetable matrix (Dubois et al., 2002). Finally, Cat-floc® has been used to improve flocculation of food solids. The mentioned components and conditions are summarized in Table 1.4.

Table 1.4 Overview of the most common components/conditions present in elution buffers used for extraction from carbohydrate/water based foods ant their respective function.

Component/condition	Detail	Function
Alkaline – neutral pH	pH 9.5 to 10.5 (alkaline) or pH 7 (neutral)	Detachment virus particles from food surface
pH buffer system	Tris(-HCl) buffer Phosphate buffer	Prevent pH drops caused by acidic fruit/vegetable juices
Beef extract	1 % to 3 % (w/v)	Facilitate flocculation of NoV on PEG
Glycine	0.05 M to 0.5 M	Reduce non-specific adsorption of protein or virus
Pectinase	180 U/300 ml to 570 U/30 ml	Prevent jelly formation of the eluate
Catfloc® TL		Improve flocculation of food solids
Soya powder	1 % (w/v)	Facilitate liberation of viruses from food surfaces

PEG Precipitation

Precipitation of the viruses is needed after elution to increase the concentration for successful molecular detection. Polyethylene glycol (PEG, 8 – 16 % w/v) has often been used for this purpose, since it easily allows the precipitation of these viruses at neutral pH and at high ionic concentrations without other organic material (Table 1.5)(Kim et al., 2008a; Lewis and Metcalf, 1988). The molecular weight of the PEG molecules (6000 Da, 8000 Da, 10000 Da or 20000 Da) did not significantly influence the recovery of NoV from strawberries or raspberries (Kim et al., 2008a).

Combining (alkalic or neutral) elution and PEG precipitation protocols with a molecular technique has generally resulted in recoveries between 5 and 90 %, depending on the food matrix and applied protocols. A study comparing different aspects of the NoV alkalic elution – PEG concentration method (using conventional RT-PCR) showed that 85% recovery of 4 × 10^4 GII.4 NoV RT-PCRU from 20 g fresh strawberry samples was possible when combining a 3% beef extraction buffer as elution buffer with 8% (w/v) PEG8000 precipitation (Kim et al., 2008a). Cheong et al. (2009a) obtained 3.9 % to 50 % recoveries when extracting 4.8 × 10^0 to 4.8 × 10^3 GII NoV RT-PCRU from 5 g of strawberries, while recoveries of 13 % have been observed when extracting 10^3 to 10^1 RT-PCRU from 30 to 100 g of fresh strawberries and raspberries. Alkalic elution combined with PEG precipitation has been selected by the CEN/TC275/WG6/TAG4 working group as the preferred method when extracting NoV from produce and soft fruits (Croci et al., 2008).

For fat/protein based foods, successful recovery of 10^3 to 10^4 RT-PCRU from hamburger was possible using this concentration approach in combination with an alkaline or neutral elution buffer, and recovery efficiencies of 10 to 24 % have been observed when detecting CaCV and NoV in whipped cream and ham, respectively. (Leggitt and Jaykus, 2000; Rutjes et al., 2006a; Scherer et al., 2010). Moreover, a study comparing two concentration methods after neutral elution showed a significant better recovery of NoV from 10 g ham samples when using PEG-precipitation (24 % recovery; detection limit 2 x 10^1 RT-PCRU/10g) compared to ultrafiltration (7 % recovery; detection limit 2 × 10^2 RT-PCRU/10g)(Morales-Rayas et al., 2010).

In shellfish, the elution-PEG precipitation (6 to 16 % w/v) approach has resulted in varying recoveries when detecting NoV from whole or digestive shellfish tissue. While some studies observed successful recovery of 5 to 22.4 NoV RT-PCRU in 1.5 g to 25 g of shellfish (digestive or whole) tissue (Atmar, 1995; Kingsley and Richards, 2001), other authors reported higher detection limits up to 3 × 10^3 RT-PCRU per 1.25 g of oyster digestive tissue (Häfliger, 1997). For HAV, successful recovery of 10 RT-PCRU has been reported from oyster digestive tissue (Sincero, 2006). Using this approach, AdV and NoV prevalence has been shown in Moroccon mussels and French oysters (Karamoko, 2005; Beuret, 2003).

Ultracentrifugation.

Ultracentrifugation has been applied to concentrate HAV and NoV virus particles eluted from shellfish, carbohydrate/water based foods and fat/protein based foods, although fewer assays have been described compared to PEG-precipitation.

For fresh strawberries and raspberries somewhat lower recoveries of 0.1 % (NoV) and 2.5 % (HAV) were obtained compared to PEG precipitation (Rzezutka et al., 2005; Rzezutka et al., 2006). Additionally, this technique has been proposed for detection of viruses from lettuce as a 10% recovery of CaCV was observed (Rutjes et al., 2006a). A likewise recovery efficiency of 10 % was observed for HAV in 25g of oysters (Casas, 2007), similar to the 3.4 %, 5.9 % and 12.5 % recoveries of the feline calicivirus (FCV) in salami, gammon and roast pork chop (Rzezutka, 2008).

The ultracentrifugation protocols require an additional purification (either high speed conventional centrifugation or 0.22/0.45 µm pore filtration) of the virus elutes, as debris and other components originating from the food samples can interfere with the ultracentrifugation protocols. During ultracentrifugation, the virus particles are precipitated by centrigational forces up to 120000 × g and 235000 × g of the filtered virus elutes (Croci et al., 2008; Rutjes et al., 2006a; Rzezutka et al., 2006). A reported advantage of this concentration technique is its consistency, but major disadvantages are the requirement of expensive equipment as well as the fact that it can be used only with virus elutes free of fruit/vegetable matter that were obtained from hard fruits or vegetables (Table 1.5)(Croci et al., 2008). A practical disadvantage of this technique is the presence of voluminous pellets that are difficult to dissolve (Rutjes et al., 2006a; Rzezutka et al., 2005).

Ultrafiltration.

Ultrafiltration of virus elutes to concentrate virus particles is based on the selection of its molecular weight (Croci et al., 2008). Appropriate filters are equipped with membrane pores permitting passage of liquids and low molecular mass particles in solution (less than 50 to 100 kDa), meaning the viruses are retained on the filter (Le Guyader et al., 2004b). Similar to ultracentrifugation, an additional purification of the virus elutes is required before ultrafiltration to prevent clogging of the filters. A published advantage of the use of ultrafiltration is that RT-PCR inhibitory components are not co-isolated with the virus particles (Table 1.5)(Rutjes et al., 2005). An increase of the recovery can be obtained by treating the filters with bovine serum albumin (BSA) or through sonication of the purified virus elute (Jones et al., 2009). A study comparing the recovery of NoV from lettuce, raspberries and ham indicated mean recoveries of 9 %, 3 % and 7% using ultrafiltration as concentration method, but PEG-precipitation resulted in significantly higher mean recoveries of 23 %, 7 % and 24 %

respectively (Scherer et al., 2010). Using an ultrafiltration based technique, recoveries of 1.7 %, 2.6 %, 17.9 % and 19.6 % were found in fresh strawberries, frozen raspberries, frozen blueberries and fresh raspberries, respectively (Butot et al., 2007). In contrast to the former study, this study found a significantly higher recovery of NoV from raspberries and strawberries using ultrafiltration compared to PEG-precipitation. Finally, a 0.1 to 1 % NoV recovery was found for lettuce using this concentration method (Rutjes et al., 2006a).

Other concentration methods.

Other strategies for concentration of eluted viruses from foods include cationic separation and immune concentration. The latter category uses magnetic beads covered either with histo-bloodgroup antigens (HBGA) type A, B, H(2) and H(3) or with type III porcine gastric mucin to bind NoV virus particles after eluting them from fresh produce samples. The bound viruses are subsequently eluted from the magnetic beads using an appropriate buffer (Morton et al., 2009; Park et al., 2008; Tian et al., 2008; Tian and Mandrell, 2006). This strategy has successfully been tested on lettuce, green onions and strawberries as well as on ham samples (Morton et al., 2009). In strawberries, GI and GII NoV were recovered with efficiencies of 14.14 and 29.50 % (Park et al., 2008), respectively, and this approach has been used to detect NoV in rolled cabbage and macaroni in a NoV food borne outbreak (Kobayashi et al., 2004). While immunomagnetic capture can increase NoV detection in various food types (oysters, strawberries, lettuce) by efficient removal of PCR inhibitors, questions have been raised regarding the long term use of HBGA due to the immunogenetic drift of the NoV (Table 1.5).

Cationic separation is based on the hypothesis that positively charged magnetic particles could be used in conjunction with a magnetic capture system for the concentration and purification of virus particles from food matrices. The purification of the viral agent is believed to occur because the negatively charged proteins of the virus capsid bind to the positively charged magnetic particles. A study by Fumian et al. (2008) showed that recovery efficiencies of 5.2 % to 56.3 % were possible using a filter-based cationic method for NoV in lettuce and Minas cheese. An automated cationic separation system named Pathatrix™ (Matrix MicroScience, Newmarket, UK) is commercially available and has been tested with varying successes. This system is promising for concentration of HAV from virus elutes of lettuce, green onions, strawberries, strawberries, deli-turkey, oysters and cake with recovery efficiencies ranging between 17.0 and 81.7 % (Papafragkou et al., 2008). However, less consistent results were obtained for NoV recovery from lettuce, strawberries, raspberries and green onions, whether or not in combination with an immune concentration step (Morales-Rayas et al., 2010; Morton et al., 2009). Similar to ultracentrifugation, expensive equipment and specialized personnel is required for this concentration method (Table 1.5).

Table 1.5 Overview of the methods for concentrating eluted virus particles and summary of reported advantages and disadvantages.

Concentration method	Advantage	Disadvantage
Polyethylene glycol precipitation	Cheap Easy to perform	pH neutralization of virus eluate necessary
Ultracentrifugation	Consistent results	Only with virus elutes free of vegetable matter Need for specialized equipment/personnel
Ultrafiltration	Removal RT-PCR inhibitors	Only with virus elutes free of vegetable matter
Immuno concentration	High specificity	Long term use unsure due to immunogenetic drift
Cationic separation	Automatization	Need for specialized equipment Inconsistent results

1.3.3.2. Acid adsorption – elution – concentration

The principles of the acid adsorption – elution – (immunomagnetic) concentration method for extraction of NoV in foods is based on the development of a technique by Sobsey et al. (1975; 1978) for extraction of enteroviruses from oyster tissue. The adsorption of the virus particles to the food tissue is accomplished by addition of an acid buffer (pH 5 to 6) while reducing the NaCl concentration under 25 mM (through addition of demineralized water). After centrifugation, the supernatans is discarded and the viruses are eluted from the food tissue pellet using either a more acidific or a neutral buffer glycine-PBS buffered solution. Finally, a concentration step is performed using either PEG or immunomagnetic beads as described for the elution-concentration protocols. The principle and more details of these elution and concentration steps are described in paragraph 1.3.3.1.

By combining acid adsorption, alkaline elution and PEG-precipitation, Love and colleagues (2008) were able to successfully extract 4.2×10^3 HAV RT-PCRU from tomato sauce while acid adsorption and subsequent immunocapture and cationic separation enabled successful detection of 10 NoV RT-PCRU from various carbohydrate/water based foods and from deli ham (Table 1.6)(Morton et al., 2009).

1.3.3.3. Direct RNA extraction

Virus extraction methods using direct RNA extraction techniques involve treatment of the food product with a guanidinium isothiocyanate-phenol-based reagent to extract RNA followed by an eventual purification/precipitation of the extracted RNA. Details of this method are described in paragraph 1.3.5.

Direct RNA extraction has successfully been evaluated on fat/protein based foods as 10^0 to 10^2 NoV RT-PCRU could be recovered in 10 to 30 g of hamburger, turkey, roastbeef, penne, tagliatelle and deli ham (Table 1.6)(Baert et al., 2008a; Boxman et al., 2007; Schwab et al., 2000). Successful recovery of 10 RT-PCRU in 0.15 g of oyster digestive tissue has been observed when using direct RNA extraction combined with shredding of this digestive tissue using zirconia beads (Table 1.6)(de Roda Husman et al., 2007). This approach has also been used to demonstrate NoV presence in oysters involved in a food borne NoV outbreak (Boxman et al., 2006). However, a comparison of an elution-concentration protocol and direct RNA extraction showed that the former was more sensitive when detecting NoV GII in mussel digestive tissue (Baert et al., 2007).

1.3.3.4. Proteinase K treatment

A recent method combining a proteinase K and heat treatment at 65°C (Comelli et al., 2008; Jothikumar et al., 2005b) has been selected by the CEN/TC275/WG6/TAG4 working group as the preferred method for extraction of the most common enteropathogenic viruses from shellfish digestive tissue (Lees, 2010). The principle is based on the attack of the capsid of the virus particles, thereby releasing the nucleic acids (Nuanualsuwan and Cliver, 2002). This approach has only been tested on shellfish foods yet (Table 1.6).

Table 1.6 Overview of different (non elution-concentration based) virus extraction methods and related detection limits or recoveries.

Protocol	**Food matrix (weight)**	**Viral agent**	**Detection limit**[a]	**Reference**
Acid adsorption Elution Concentration	Deli ham, lettuce, green onions, strawberries (25g)	NoV	10^2 – 10^4 RNA copies	(Morton et al., 2009)
	Tomato sauce, strawberries (30g)	HAV	14 – 33 PFU	(Love et al., 2008)
Direct RNA extraction	Oysters (0.15 g)	NoV	10 RT-PCRU	(de Roda Husman et al., 2007)
	Oysters (5-50 g)	HAV	8PFU	(Cromeans et al., 1997)
	Hamburger, turkey, roastbeef (30 g)	NoV/HAV	10^2 RT-PCRU	(Schwab et al., 2000)
	Penne tagliatelle salad (10 g)	NoV	10^2 RT-PCRU	(Baert et al., 2008a)
	Deli ham (10 g)	NoV/PV	1 RT-PCRU	(Boxman et al., 2007)
Proteinase K	Mussels (n = 10-15)	NoV (GI/GII)		(Comelli et al., 2008)
	Oysters	NoV		(Jothikumar et al., 2005b)
	Oysters	NoV GI NoV GII	10^2 RNA copies[b]	(Le Guyader et al., 2009)

[a] by (real-time) RT-PCR. [b] observed recovery efficiencies: 14.7 – 20.5 % (NoV GI) and 5.3 – 33.6 % (NoV GII)

1.3.4. Purification virus eluate/concentrate or extracted RNA

Purification (removal of food debris and inhibitory substances) of the eluted or concentrated viruses is in most protocols performed by either filtering or by treatment with Freon 113, Vertrel® XF or chloroform:butanol. This is particularly important when a molecular virus detection method is subsequently used.

Due to its physical and chemical properties, Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane) is able to extract lipids and lipid bilayers without extracting proteinaceous (polar) material such as non-enveloped viruses (Kobayashi et al., 2004; Liebermann and Mentel, 1994; Rieht et al., 1993). This reagent has therefore been used for the purification of eluted viruses in food, but because Freon has been implicated in ozone layer depletion and because of rising environmental concerns its use has been progressively eliminated (Taylor, 1996). Vertrel® XF (1,1,1,2,3,4,4,5,5,5-decafluoropentane), an ecologic Freon substitute developed by DuPont has provided similar results when purifying NoV RNA from faecal samples (Mendez et al., 2000). Its use has been successfully evaluated in several protocols for virus extraction from fat/protein based foods (Fumian et al., 2009; Papafragkou et al., 2008). The use of Vertrel® XF has not been investigated in protocols for virus extraction in shellfish yet.

A second alternative for Freon is organic solvent purification such as chloroform:butanol purification, which produced better results compared to Freon when recovering HAV from oysters (Mullendore et al., 2001). This purification method has been used for direct RNA extraction protocols in shellfish and when detecting NoV from carbohydrate/water based foods (Baert et al., 2007; Baert et al., 2008a)

Filter-purification of the eluent has been performed by cheesecloth filtering (Leggitt and Jaykus, 2000) or through 0.45 µm and 0.20 µm filters (Scherer et al., 2010).

1.3.5. RNA extraction/purification

Since most virus extraction protocols are based on the (acid adsorption,) elution and concentration of virus particles from food matrices, an RNA extraction/purification step involving (1) a lysis step to release the nucleic acids trapped in the viral capsid and (2) a subsequent RNA extraction/purification step is required. On the other hand, direct RNA extraction protocols (described for virus extraction from shellfish and fat/protein based foods) consist already of the direct extraction and purification of RNA from the food matrix. However, purification of this extracted RNA can be necessary to remove inhibitory substances, as well as to concentrate the extracted RNA.

Lysis step.

Two major strategies have been described for the lysis step.

Firstly, the use of a heat release step consisting of a 5 minute incubation at 99 °C or 95 °C to destabilize the viral capsid and release the nucleic acids has successfully been used in clinical, shellfish and water samples (Bae and Schwab, 2008; Dix and Jaykus, 1998; Schwab et al., 1997). While this is a simple and efficient method, a disadvantage of this technique is the simultaneous release of nucleases. In particular ribonucleosidases (RNases) are a problem, due to their resistance for such heat treatments. Therefore, processing of the RNA should be performed immediately (Atmar, 2006).

Secondly, an approach based on the combined use of guanidine isothiocyanate (GITC) and phenol has been published frequently for extraction of viral RNA from clinical samples as well as carbohydrate/water based foods, fat/protein based foods and shellfish (Baert et al., 2007; de Cal et al., 2007; Dove et al., 2005; Guevremont et al., 2006; Papafragkou et al., 2008). Guanidine hydrochloride is a less used alternative for GITC. The combination of GITC and phenol allows the maintaining of the integrity of the extracted RNA while disrupting/lysing cells and solubilizing cell components (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006). A proof of the robustness of this method is its use as RNA extraction technique in other matrices such as plant, fungi and animal tissues (Portillo et al., 2006; Que et al., 2008; Ye et al., 2009). Other advantages of this technique are its cheapness as well as its availability as commercial solutions such as TRIzol® reagent (Invitrogen, Merelbeke, Belgium), TriReagent (Ambion, Austin, TX) and RNAzol® (Biotex Laboratories, Houston, TEX). Despite its cheapness and robustness, phenol and GITC are toxic substances and thus require the availability of equipment such as fume hoods when handling. A study comparing several RNA extraction methods in clinical samples and shellfish extracts showed that methods based on the use of GITC (and phenol) were less sensitive in clinical samples, but more sensitive in shellfish samples compared to resin or immune based RNA extractions (Arnal et al., 1999).

Cetyl trimethylammonium bromide (CTAB) and proteinase K are both reagents that are used to facilitate the lysis step of viral capsids, especially when detecting viral agents in shellfish (Atmar et al., 1995; Le Guyader et al., 2009; Sincero et al., 2006).

RNA isolation/purification

After lysis of the viral capsid and release of the viral RNA, an RNA purification step is required, except for heat-released viral RNA which is directly used for molecular detection. No reports of RNA degradation due to RNase activity has been reported using this method in clinical and water samples, although RNA extracts from clinical samples had to be diluted in some cases for successful detection (Bae and Schwab, 2008; Schwab et al., 1997). Most

RNA purification protocols are either based on treatment with organic solvents or on the use of (magnetic) silica.

During organic solvent RNA purification, chloroform is used for separation of the lysate in two phases. While the aqueous phase contains the RNA, most of the DNA and proteins are collected in the organic phase. The RNA is subsequently precipitated and purified with isopropanol and ethanol, respectively. The main advantage of this method is its cheapness, but the use of the toxic chloroform is required (Chomczynski and Sacchi, 2006). Organic solvent purification of extracted viral RNA has been used in clinical and food samples (Baert et al., 2008a; Boxman et al., 2006; Guevremont et al., 2006; Kingsley and Richards, 2001; Leggitt and Jaykus, 2000).

A more recently developed RNA purification method is based on the nucleic acid-binding properties of silica particles or diatoms (Boom et al., 1990). In combination with a high concentration of GITC, silica particles and diatoms have the ability to bind nucleic acids efficiently. After a washing step, these nucleic acids can be released using an appropriate elution buffer. While diatoms are only used occasionally, silica particles are currently commercially available as (magnetic) beads or as filter columns. It has been reported that the addition of a silica binding step is particularly helpful to remove inhibitors associated with processing food samples (Dubois et al., 2007; Le Guyader et al., 2004b; Rutjes et al., 2006a).

Commercial kits

A wide variety of commercial RNA extraction and purification kits and solutions are currently available, but the majority of these kits is based on the lysis-purification principle as described above. Kits that are often used for extraction and purification of RNA from viruses are the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), the QIAamp RNeasy kit (Qiagen), the RNAfast II kit (Molecular Systems, San Diego, CA), the Total Quick RNA isolation kit (Talent™, Italy) and the NucliSens kits (BioMérieux, Marcy l'Etoile, France).

1.3.6. Molecular detection of viral RNA

Molecular detection of food borne viruses can be performed using either amplification-based or non amplification based methods and a variety of assays are available for both categories. Assays based on hybridization with RNA/DNA probes are the most used non amplification based methods and have been applied for virus detection in clinical samples (Wang et al., 2006). However, typically 10^4 to 10^5 viral genomic copies are needed to yield unambiguous results which means this technique is not suitable for virus detection in food and environmental samples due to low viral numbers (Sair et al., 2002; Wernars and Notermans, 1990).

A variety of amplification based methods for detection of (viral) RNA have been described including “nucleic acid sequence based amplification (NASBA)(Compton, 1991)”, “self-sustained sequence replication (3SR)(Fahy et al., 1991)” and “transcription amplification system (TAS) (Kwoh et al., 1989)” All these techniques are based on the initial conversion of RNA to copy DNA (cDNA) using a reverse transcriptase followed by the *in vitro* transcription of this cDNA using an RNA polymerase. However, the most widely used technique for amplification of RNA is “reverse transcriptase polymerase chain reaction (RT-PCR)” and combines a reverse transcription step with the polymerase chain reaction (PCR), an *in vitro* technique for enzymatic amplification of target nucleic acid sequences using a specific pair of primers and a heat-stable DNA polymerase (Saiki et al., 1988).

1.3.6.1. Reverse transcription

The conversion of RNA to copy DNA (cDNA) is performed by reverse transcription (RT). Initially, the RNA is hybridized with either random hexamers (random six-nucleotide sequences), oligodT primers or specific primers. The formed RNA-DNA hybrid subsequently functions as template for the reverse transcriptase forming a RNA-DNA heteroduplex which is denaturated through a heat treatment.

The use of random hexamers, oligodT primers and specific primers have been thoroughly compared, but no consensus could be found for RT of (viral) RNA. Although the use of random hexamers can result in a reduced linearity of product amplification an RT-PCR assay, its use has also resulted in a higher cDNA yield compared to oligodT or specific priming (Lekanne Deprez et al., 2002; Zhang and Byrne, 1999). In contrast, Jothikumar and colleagues (2005b) found that specific priming caused a higher cDNA yield compared to random hexamers.

The RT can be performed separately or in combination with the subsequent PCR (using an appropriate temperature program). The latter is called “one-step RT-PCR” and although it is more convenient due to reduced handling time, a reduced sensitivity has been noticed (Battaglia et al., 1998; Bustin, 2002). In contrast, the former setup called “two-step RT-PCR” is more laborious, but allows the use of the prepared cDNA for other purposes such as confirmation or NoV genotyping.

1.3.6.2. NoV RT-PCR primer development.

Sequencing of the NoV genome by Xi et al. (1990) lead to the development of RT-PCR assays for detection of NoV and random target sequences were initially chosen for amplification (DeLeon et al., 1992; Jiang et al., 1992). Comparison of several NoV genomes showed in 1993 that a great diversity existed between the NoV genomes as over 30 %

sequence difference could be observed between different NoV strains (Green et al., 1993). This lead to the classification of NoV in two genogroups initially named Norwalk-like viruses and Snow Mountain like viruses, later renamed genogroup I and II, respectively (Wang et al., 1994). The subsequent development of broadly reactive NoV detection RT-PCR assays targeted either the RdRp gene situated on the 3' side of the first ORF in the NoV genome (Ando et al., 1995; Atmar et al., 1995) or two conserved regions on the 3' side of the gene encoding for the VP1 protein (Green et al., 1995; Noel et al., 1997). These regions were later renamed as region A, C and E, respectively (Mattison et al., 2009b; Vinje et al., 2004). In 2000, the junction between ORF1 and ORF2 of the NoV genome was recognized as one of the most conserved regions (Fig. 1.8) and was targeted by most of the subsequent developed RT-PCR assays, although GI and GII NoV required separate primer pairs (Jothikumar et al., 2005b; Kageyama et al., 2003; Wolf et al., 2007). The CEN/TC275/WG6/TAG4 working group has also selected the ORF1/ORF2 junction as target for RT-PCR detection of NoV (Le Guyader et al., 2009). A recent evaluation of different RT-PCR NoV detection assays targeting the ORF1/ORF2 junction using a variety of NoV strains circulating between 2007 and 2009 in Canada confirmed that this target still allows amplification of a broad range of NoV genotypes.

A overview of some described primers and hydrolysis probes for real-time PCR assays targeting the ORF1/ORF2 overlap in the NoV genome is shown in Fig. 1.9.

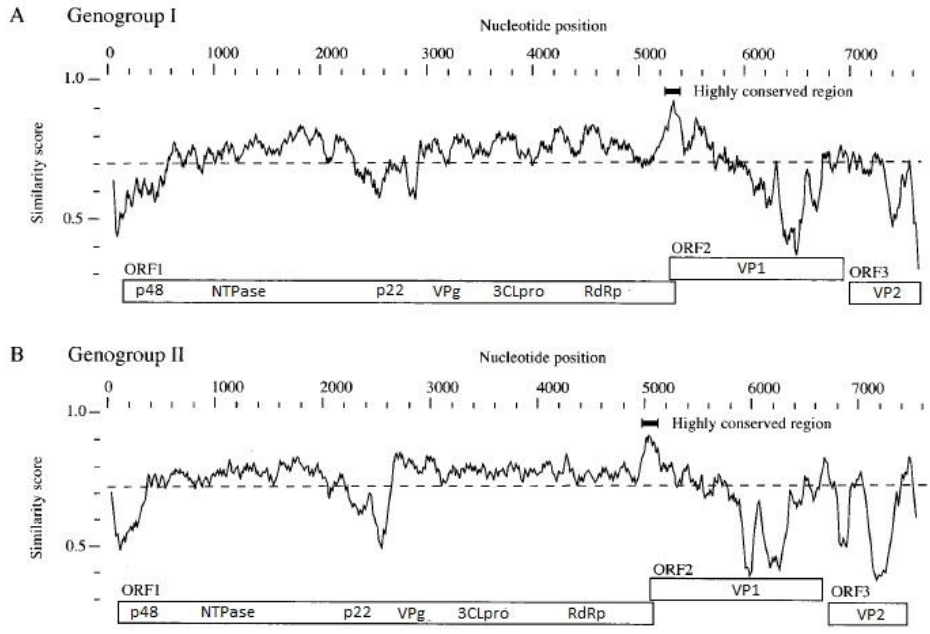


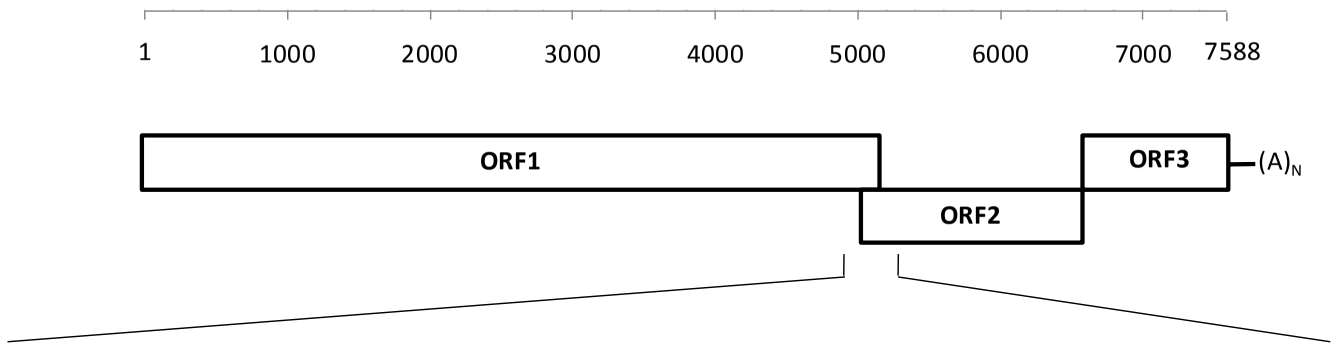
Fig. 1.8 Conservation of the genome of genogroup I and II NoV based on four NoV GI strains (A) and 10 NoV GII strains. Thick lines depict the most conserved regions. The average similarity score within each genogroup is represented as a dotted line. Source: Kageyama et al., 2003.

1.3.6.3. Conventional PCR.

The general principle of PCR in its simplest form was described in the late 1980's (Saiki et al., 1988) and has been frequently used for detection of food borne pathogens (Rijpens and Herman, 2002). The performance of PCR assays can be influenced by the annealing temperature as well as by the degeneration of the primers. A high annealing temperature and the use of specific (not degenerated) primers assures specific amplification of the selected target, but makes the development of a broadly reactive PCR for detection of various NoV strains difficult. On the other hand, while a reduced annealing temperature and the use of degenerated primers (by including inosines or allowing combinations of primers) allow the detection of a broad range of NoV, aspecific amplicons can be generated, in particular when detecting NoV in food samples (Atmar, 2006; Baert et al., 2009b). The same principle also counts for real-time PCR primer development.

Visualization of the amplicons can easily be performed by agarose or polyacrylamide gel electrophoresis, although the occurrence of aspecific amplification can lead to bands that are of the expected size but are not virus-specific (Atmar et al., 1996). Use of restriction or hybridization assays or sequencing of the amplicons can provide additional reassurance of the specificity of the bands.

An improvement of the sensitivity can be obtained through hemi-nested and nested RT-PCR by performing a second round PCR on the amplicons using respectively one or two primers flanking the inside of the primers used in the first round PCR. The use of nested RT-PCR for detection of NoV in shellfish was found to be 10 to 1000 times more sensitive than single round RT-PCR and Häfliger et al. (1997) developed hemi-nested RT-PCR systems for detection of enterovirus, RoV, HAV, and GI and GII NoV. Burkhardt et al. (2002) developed a compartmentalized tube-within-tube device to combine the RT-PCR reaction with nested PCR for a calicivirus strain (San Miguel sea lion virus, serotype 17). In combination with the reduced risk of carry-over contamination by not opening the reaction tubes between the two round of the PCR, the handling time was reduced as well. In general, the use of nested RT-PCR has been proven successful for detection of enteric viruses in water, food and clinical samples (Campos et al., 2008; Love et al., 2008; O'Neill et al., 2001).



GI NoV

5271 **5385**

CAGGATGGCAGGCCATGTTCCGCTGGATGCGCTTCCATGACCTCGGATTGTGGACAGGAGATCGCGATCTTCTGCCCGAATTCGTAAATGATGATGGCGTCTAAGGACGCTACA

(QNIF4) CGCTGGATGCGNTTCCAT	TGGACAGGAGAYCGCRATCT (NVGG1p)	CATTTACTACTACCGCAGATTCC (NV1LCR)	(CEN/TC275/WG6/TAG4
(JJ1VF) GCCATGTTCCGITGGATG	TGTGGACAGGAGATCGCAATCTC (JJV1P)	TACTACTACCGCAGATTCCT (JJ1VR)	(Jothikumar et al.,
(NV192) GCYATGTTCCGCTGGATGC	TGGACAGGRGATCGCRATCT (NV65a)	ACTACTACCGCAGATTCCTGC (NV193)	(Hoehne & Schreier,
(COG1F) CGYTGGATGCGNTTYCATGA	(RING1(a)-TP) AGATYGCGATCYCCTGTCCA	CAYTTACTACTACCGCAGATTC (COG1R)	(Kageyama et al.,
	(RING1(b)-TP) AGATCGCGGTCTCCTGTCCA		

GII NoV

4997 **5108**

CCCAGACAAGAGCCAATGTTCAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGCTTTGTGAATGAAGATGGCGTCGAATGACGCCAA

(QNIF2d) ATGTTCAGRTGGATGAGRTTCTCWGA	AGCACGTGGGAGGGCGATCG (QNIFS)	ACACTTACTTCTACCGCAGCT (COG2R)	(CEN/TC275/WG6/TAG4)
(JJV2F) CAAGAGTCAATGTTTAGGTGGATGAG	TGGGAGGGCGATCGCAATCT (RING2-TP)	ACACTTACTTCTACCGCAGCT (COG2R)	(Jothikumar et al.,
(NV107a) AGCCAATGTTCAGATGGATG	TGGGAGGGCGATCGCAATCTGGC (TM3A)	CACTTACTTCTACCGCAGCT (NV117)	(Hoehne & Schreier,
(COG2F) CARGARBCNATGTTYAGRTGGATGAG	TGGGAGGGCGATCGCAATCT (RING2-TP)	ACACTTACTTCTACCGCAGCT (COG2R)	(Kageyama et al.,

Fig. 1.9 Overview of some real-time PCR primers and hydrolysis probes designed in the ORF1-ORF2 overlap of NoV GI and GII. NoV GI and GII sequences shown and deduced from EMBL/Genebank accession numbers M87661 and X86557, respectively. Names of primers and hydrolysis probes are given between brackets in front or behind the primer or probe sequence.

1.3.6.4. Real-time PCR.

In the early 1990's, Higuchi and colleagues. (1992; 1993) lead the way for development of real-time PCR by construction of a system based on the use of the intercalating dye ethidium bromide. In this system, ethidium bromide was included in the PCR reaction, the thermal cycler was irradiated with UV light, and fluorescence was detected with a charge-coupled device (CCD) camera. Amplification produces increasing amounts of double stranded DNA (dsDNA), which binds ethidium bromide, resulting in an increase of fluorescence. Nowadays, improved chemistries have lead to the use of real-time PCR for multiple purposes such as detection/quantification of pathogens and gene expression studies.

Real-time PCR chemistries can be divided in two main categories. Sequence independent chemistries are based on the use of intercalating dyes while sequence dependent chemistries are based on the phenomenon of fluorescence energy transfer (FRET). During the latter phenomenon the energy from an excited fluorophore is transferred to an acceptor moiety at distances up to 70–100 Å and emitted either as light at a specific (non detected) wavelength or as heat. As a result, the emission of the fluorophore is quenched (Rijpens and Herman, 2002; Selvin, 1995).

For detection of NoV in clinical, food and environmental samples, real-time PCR assays are considered to be the gold standard (Baert et al., 2007; Jothikumar et al., 2005b; Park et al., 2008; Wolf et al., 2007). Most important reasons for this choice are (1) the low detection limit in comparison to conventional RT-PCR and other molecular methods (Beuret, 2004), (2) the absence of post-PCR processing and finally (3) the possibility of quantification (Mackay et al., 2002; Niesters, 2002). The low detection limit (≤ 10 target copies) is necessary because of the low viral concentration in environmental and food samples as well as because of the low infectious dose of NoV.

Sequence independent chemistries

Examples of commercially available dyes intercalating in the minor groove of double stranded DNA (dsDNA) are Sybr Green I (Fig. 1.10), SYTO-9, SYTO-13 and SYTO-82. These dyes are 10- to 100-fold more fluorescent when bound to dsDNA compared to unbound status (Fig 1.11)(Monis et al., 2005; Zipper et al., 2004). Therefore, when added to PCR reaction mixes, the amplification of the target sequence can be followed by fluorescence measurement during each primer extension phase of each PCR cycle. Detection of these intercalating dyes is

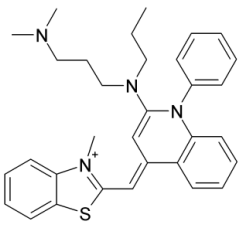


Fig. 1.10 Chemical structure of Sybr Green I, a frequently used intercalating dye for real-time PCR assays.

performed at their maximal emission wavelength, which can vary according the used dye. Maximal emission wavelengths of the exampled dyes are 494 nm (Sybr Green I), 498 nm (Syto-9), 509 nm (Syto-13) and 560 nm (Syto-82).

A disadvantage of this technique is the fact that aspecific amplicons and primer dimers will result in a positive fluorescent signal although this problem can be greatly resolved by performing a melting curve analysis after amplification. During this analysis, the temperature in the thermal chamber is slowly raised and the fluorescence in each tube is measured. As soon as the dsDNA starts to denature, the intercalating dye is released which results in a fluorescence decrease. Because each dsDNA product has its own characteristic melting temperature (Tm), depending on its length and guanosine-cytosine (GC) content, melting curve analysis can be compared with analyzing a PCR amplicon by length in gel electrophoresis (Rijpens and Herman, 2002). Another disadvantage of this sequence independent system is its inability for multiplexing. However, the biggest advantage of this technique as well as the main reason for its widespread use is the relatively low cost compared to sequence dependent real-time PCR systems. Sybr Green I based real-time PCR assays have been described for detection of AdV, EV, HAV, HEV and NoV (Brooks et al., 2005; Donia et al., 2005; Orru et al., 2004; Pang et al., 2004; Richards et al., 2004; Watanabe et al., 2005).

A more recent improvement of the melting curve analysis is called “high resolution melting” (HRM) and makes use of (1) instruments allowing more precise temperature increase and data acquisition and (2) of fluorescent dyes such as LC Green® and LC Green Plus® , ResoLight® and EvaGreen® with improved dsDNA binding saturation properties (Gundry et al., 2003; Wittwer et al., 2003). HRM is a powerful technique allowing the detection of mutations, polymorphisms and epigenetic differences in dsDNA samples and has successfully been used to differentiate different NoV genotypes after amplification with broad range NoV primers (Tajiri-Utagawa et al., 2009). Nowadays, several platforms combining real-time PCR and HRM are commercially available (Herrmann et al., 2006; Herrmann et al., 2007).

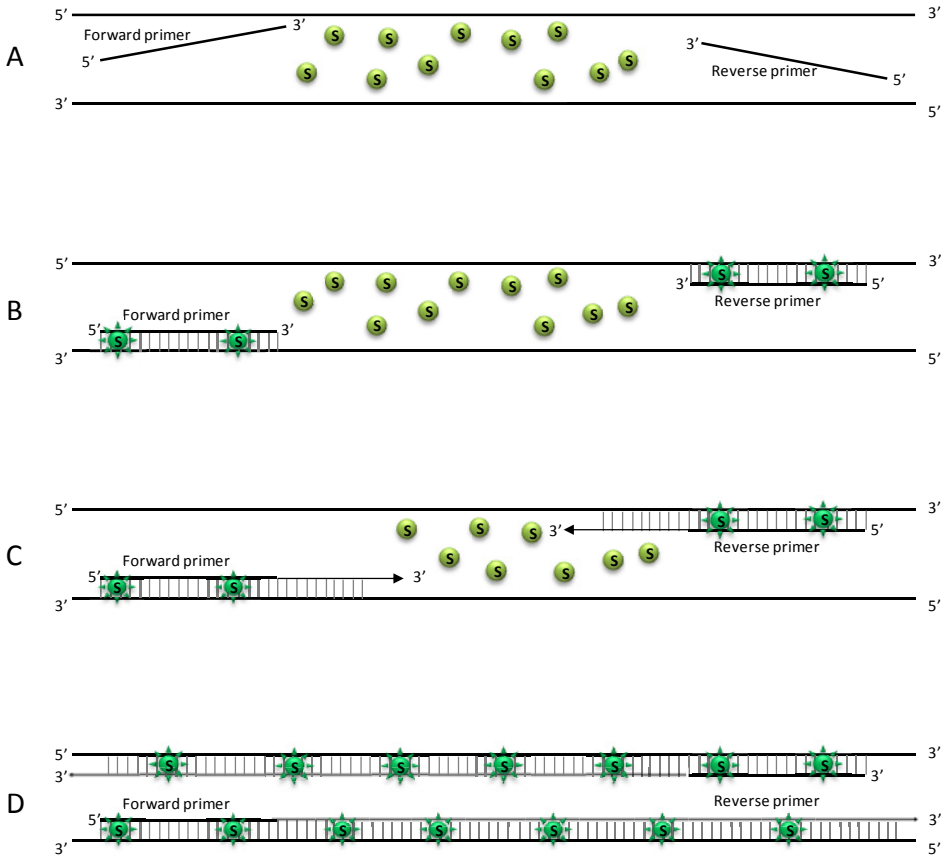


Fig. 1.11 Principle of intercalating dyes used in real-time PCR assays. Binding of primers to the template occurs during the annealing phase of the PCR reaction (A and B). Subsequently, the primer-template DNA hybrid is elongated by a DNA polymerase (C and D). During and after annealing and elongation, an intercalating dye "S" can bind to the formed double stranded DNA molecules, thereby increasing fluorescence.

Sequence dependent chemistries

A variety of sequence dependent real-time PCR chemistries are available, although only a few have been used for detection of enteric viruses. Most used systems are the 5' nuclease assay, the hybridization probe chemistry and the molecular beacon technology.

The 5' nuclease assays make use of two conventional primers and one dual-labeled hydrolysis probe (5' side: reporter dye, 3' side: quencher moiety; fluorescent signal

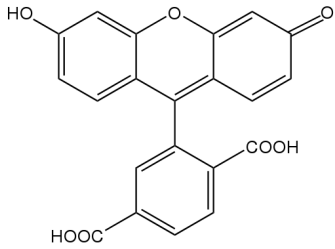


Fig. 1.12. Chemical structure of 6-carboxyfluorescein (6-FAM), a frequently used reporter dye added to the 5' side of hydrolysis probes used for real-time PCR assays.

quenched by FRET transfer). Frequently used 5' reporter dyes are 6-FAM (Fig 1.12), VIC, HEX and NED, although a broad variety of dyes are currently available. Quenching of the fluorescent signal can be performed by use of a 3' moiety such as TAMRA, that emit a fluorescent signal at a different wavelength or by use of heat releasing quenchers such as Deep Dark Quenchers® or Black Hole Quenchers®.

After annealing of the primers and hydrolysis probe, the probe is cleaved during the primer extension due to the 5' nuclease activity of the DNA polymerase. The fluorophore and quencher are hereby separated, allowing the fluorophore to emit a fluorescent signal (Fig. 1.14). This way, an increase of fluorescence is measurable every cycle in case of amplification (Holland et al., 1991).

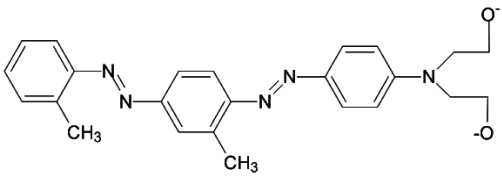


Fig. 1.13. Chemical structure of Black Hole Quencher 1, a frequently used quenching moiety added to the 3' side of hydrolysis probes used for real-time PCR assays.

It is worth noticing that the annealing temperature of the hydrolysis probe is approximately 10°C higher compared to the primers, assuring the binding of the hydrolysis probe to the target sequence before extension of the primers. Most likely due to its pioneering role, the majority of sequence dependent real-time PCR assays have used this chemistry (Kubista, 2004). Assays based on this chemistry have been developed for detection of all major enteric viruses (AdV, enteroviruses (EV), HAV, HEV, NoV)(Butot et al., 2010; He and Jiang, 2005; Jothikumar et al., 2005a; Jothikumar et al., 2006; Park et al., 2008; Petitjean et al., 2006). This chemistry is also often referred to as "TaqMan" real-time PCR technology, based on its commercial name by Applied Biosystems. An improvement of the conventional TaqMan® probe is the use of the non-fluorescent minor groove binding quencher (NFMGBQ), which forms stable duplexes with single-stranded DNA targets, allowing shorter probes to be used for hybridization based assays (Kutyavin et al., 2000). This can be useful when amplifying sequences in rapidly evolving genomes such as the NoV genome.

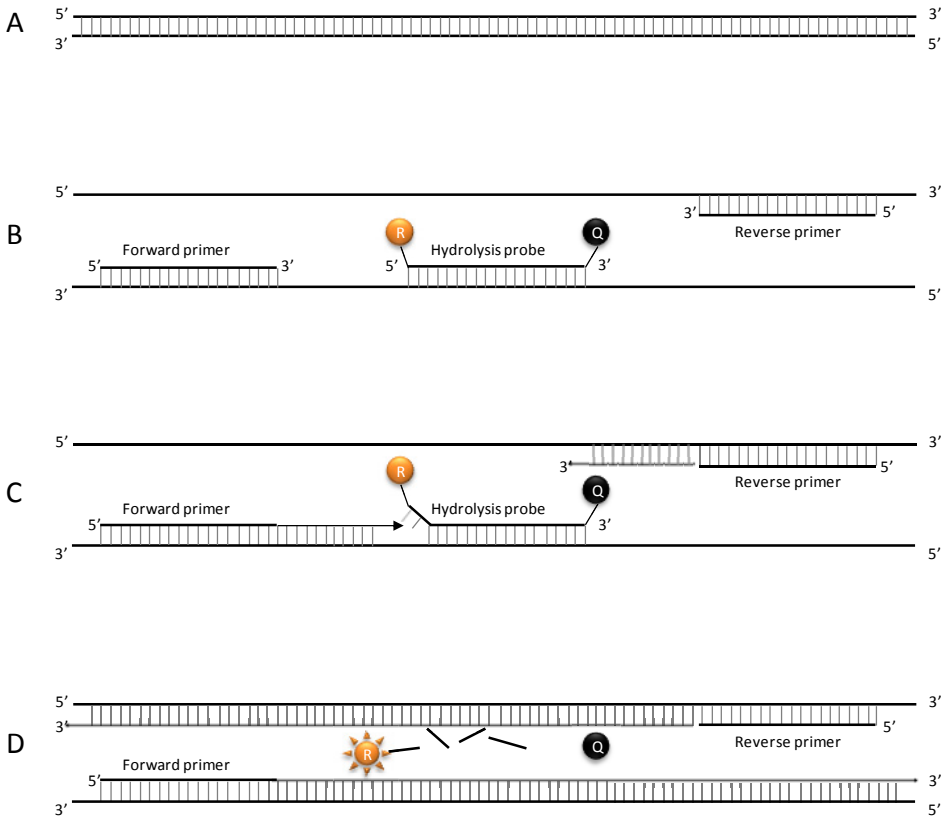


Fig. 1.14 Principle of the 5' nuclease assay used for real-time PCR. Double stranded DNA is denatured at temperatures ~95°C (A). Subsequently, primers and hydrolysis probes can bind to the single strand DNA molecules (B). In this state, the fluorescent signal of the reporter dye "R" is quenched by the moiety "Q" through FRET transfer. During extension of the primer-target DNA hybrid (C), the hydrolysis probe is cleaved. This results in the separation of reporter dye "R" and quenching moiety "Q", allowing detection of the fluorescent signal of the reporter dye (D).

Alternative sequence dependent real-time PCR chemistries are available but are rarely used for detection of enteric viruses, in particular in foods.

Hybridization probe assays (also called LightCycler chemistry assays) and molecular beacon assays are two alternatives, with the latter chemistry being used mainly in nucleic acid sequence based amplification (NASBA) assays for detection of NoV (Lamhoujeb et al., 2008; Rutjes et al., 2006b) and HAV (Abd et al., 2004; Yeh et al., 2008). The main advantage of hybridization probe assays, their ability for very specific priming since the hybridization of two probe sequences and two primers is required, is also a disadvantage for detection of several strains or genotypes of a virus. This chemistry is therefore rather used for specific detection of viral pathogens such as Epstein-Barr virus and hepatitis B virus (HBV) in clinical samples

(Krumbholz et al., 2006; Leb et al., 2004; Pham et al., 2004). Other alternative real-time PCR assays use a concept similar to the hairpin probes, but the label becomes irreversibly incorporated into the PCR product, creating a so-called "self-fluorescing amplicon". Commercially available systems are the Sunrise system (commercial name: Amplifluor™ hairpin primers)(Winn-Deen, 1998), the Eclipse™ system (Afonina et al., 2002), LUX™ primers (Lowe et al., 2003) and the Scorpion primers (Nazarenko et al., 2002). However, these technologies have not been used for detection of enteric viruses yet, except for the LUX™ primers, which have been used for NoV and RoV detection (Nordgren et al., 2008; Nordgren et al., 2010a).

1.3.6.5. Multiplex PCR

An possibility of PCR is multiplexing, whereby two or more different target sequences are amplified using multiple primer pairs in a single PCR reaction (Chamberlain et al., 1988). The advantages of this setup are clear as – besides the reduced handling time and cost – samples can be evaluated for multiple targets in a single reaction tube.

However, a successful multiplex (RT-) PCR, whether or not in real-time format, requires thorough evaluation of a number of aspects. Firstly, the addition of the extra primers and probes should not result in a loss of test performance, such as reduced sensitivity or the formation of non-specific products. Other factors such as robustness, reproducibility and, if relevant, linearity and quantification accuracy should also be assessed (Gunson et al., 2008). Secondly, a reliable simultaneous amplification of more than one target should be possible, even when these targets are present in different concentrations (Ishii et al., 2007; Molenkamp et al., 2007). This is in particular important when multiplexing quantitative (real-time) PCR assays.

Multiplex conventional RT-PCR assays have been used for simultaneous detection of RoV, NoV, SaV and AdV in faeces samples (Kittigul et al., 2009; Yan et al., 2003). Due to the increased number of real-time PCR fluorophores (covering a broad emission spectrum), multiplex real-time (RT-)PCR assays have been developed as well. Assays for simultaneous detection of various enteric viruses have been described in recent literature (Beuret, 2004; Hoehne and Schreier, 2006; van Maarseveen et al., 2010; Wolf et al., 2010).

1.3.6.6. Alternative molecular detection methods

A published alternative for (real-time) RT-PCR is nucleic acid sequence based amplification (NASBA), a technique capable of isothermal amplification of RNA. Advantages of this technique compared to RT-PCR are (1) the isothermal protocol which avoids the requirement for a thermocycler. However, use in real-time format of this system is somewhat more difficult since the use of hybridization probes or molecular beacons is required, which is difficult for

most food borne viruses. Nevertheless, this technique has been suggested in a number of NoV detection methodologies and studies have shown that RT-PCR and NASBA have similar sensitivities (Houde et al., 2006; Jean et al., 2004; Moore et al., 2004).

A second substitute for (real-time) RT-PCR is reverse transcription and loop-mediated isothermal amplification (RT-LAMP), an isothermal DNA amplification method (Notomi et al., 2000). However, the need for different primer sets targeting different genomic regions might hinder development of long-term use assays due to the immunogenetic drift in the NoV genome. Nonetheless, this promising new technique has already been used for detection of NoV (Fukuda et al., 2006; Fukuda et al., 2007; Li et al., 2009; Yoda et al., 2007), HAV (Yoneyama et al., 2007) and enteroviruses (Arita et al., 2009).

1.3.7. Genotyping of NoV

Currently, classification of NoV into genotypes is based on phylogenetic analysis of the complete capsid amino acid sequence (Ando et al., 2000; Vinje and Koopmans, 1996; Zheng et al., 2006).

However, most NoV genotyping data are currently based on sequencing of certain genomic regions after RT-PCR amplification. While genomic regions with a high conservation grade (such as the ORF/ORF2 overlap) are used for detection of NoV, genomic regions with a lower conservation grade that are able to discriminate NoV genotypes are used for genotyping. In detail, the polymerase gene (regions A and B) or the gene encoding the major capsid protein VP1 (regions C, D, and E; (Anderson et al., 2003; Ando et al., 2000; Gonin and Couillard, 2000; Kojima et al., 2002; Noel et al., 1997; Vennema et al., 2002; Vinje et al., 2004) have been used to genotype NoV strains (Fig. 1.12)(Mattison et al., 2009b). The use of these different genotyping regions, combined with different primer sets per region has resulted in scattered NoV genotyping data which are often difficult to compare (Vinje et al., 2003). Some difficulties may occur when trying to genotype NoV detected in food samples. Partial genome sequencing does not only require amplification of a region which is sufficient in size, but also requires sufficient amount of target material. The latter can be problematic when genotyping NoV present in foods, since in most cases only low concentrations are present.

Furthermore, like all RNA viruses, NoV exhibit great genetic variability and are thought to evolve rapidly (Kroneman et al., 2008b; Siebenga et al., 2007b), which has lead to the formation of various recombinant NoV strains (Bull et al., 2007; Han et al., 2004; Nayak et al., 2009). Recombination has been shown to occur most frequently at the junction between ORF1-ORF2 in different members of the *Caliciviridae* family (Bull et al., 2007; Coyne et al., 2006; Hansman et al., 2007; Mathijs et al., 2010).

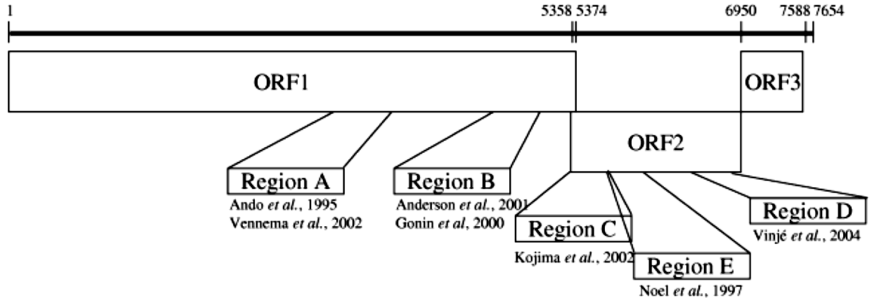


Fig. 1.15 Schematic representation of the NoV genome and the positions of several representative regions (regions A to E) that have been used for genotyping. Numbers refer to positions in the Norwalk GI.1 virus genome (GenBank accession number M87661). Source: Mattison et al., 2010.

1.3.8. Quality controls

In general, quality control of methods for detection of viruses in food and in clinical samples consists of the use of adequate positive controls (to notice false negative results due to inhibition of the RT-PCR or because of inefficient virus extraction/RNA purification) and negative controls (to detect false positive results caused by contamination) throughout different steps of the viral detection procedure that are considered critical for correct detection or quantification.

1.3.8.1. Control strategies

A recent study proposed a strategy for controlled detection of HAV in shellfish which included controls for the real-time RT-PCR reaction, as well as for the complete detection/quantification procedure (Fig. 1.16)(Costafreda et al., 2006). The approach described by Costafreda et al. combined the use of a process control with a reverse transcription control. The first step of this detection strategy consisted of the addition of a known concentration of a genetically modified mengovirus (vMC_0; see paragraph 1.3.8.2) as process control to the sample. After purification of the RNA, three separate subsamples of the purified RNA were analyzed. To subsample 1, a known concentration of a run-off ssRNA molecule covering HAV primer and hydrolysis probe binding sites was added as reverse transcription control (RTC) to estimate the efficiency of the reverse transcription and real-time PCR. In Fig. 1.16 the RTC was called ssRNA internal control (ssRNA IC). To subsample 2, no RTC was added. Prepared cDNAs of subsamples 1 and 2 were subjected to the real-time PCR assay for quantification of HAV (in subsample 1, the number of added ssRNA RTC HAV genomic copies was subtracted from the total number of detected HAV genomic

copies). In subsample 3, quantification of the vMC_0 process control was interpreted as an estimation of the HAV RNA extraction efficiency. The actual number of detected HAV genomic copies was then calculated based on this extraction efficiency. In contrast to the above described controlled detection strategy, many control systems assess only the molecular detection step when detecting viruses in foods, water and clinical samples (Casas et al., 2007; Dingle et al., 2004; La Rosa et al., 2007).

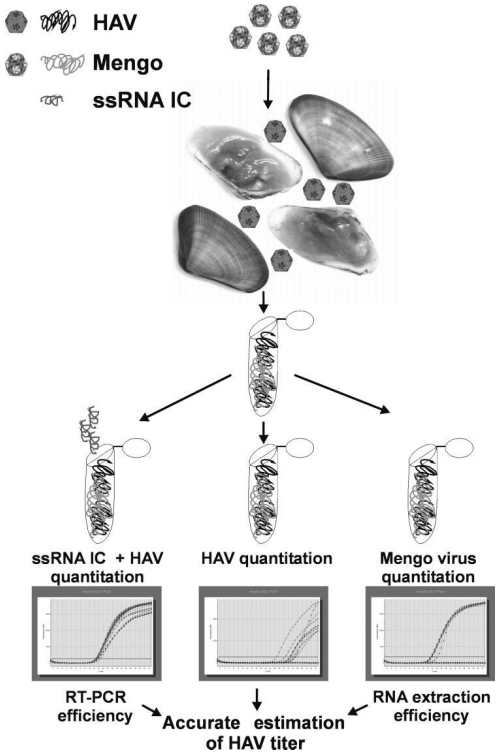


Fig. 1.16 Proposed standardized procedure for an accurate estimation of HAV genome copies in food or clinical samples. IC, internal control. Source: Costafreda et al., 2006.

1.3.8.2. Positive controls (Fig. 1.6)

Process controls (PC)

To date, the bottleneck of methods for detection of enteric viruses in food and environmental samples is situated at the virus extraction step due to the use of specialized and lengthy in-house methods. Although these virus extraction methods can yield reliable results, it is important to implement a process control to indicate the absence of inhibition, as well as to estimate the extraction efficiency of the used protocol. Process controls can be added to the analyzed sample as internal process controls (IPC) or in a parallel analyzed sample within the same setup as an external process control (EPC) (Croci et al., 2008). While the use of an

IPC is more labor dependent, requiring the addition of an extra molecular detection assay, it allows the determination of the extraction efficiency from every individual sample as this can differ with respect to viral recovery and inhibitor removal (Hoorfar et al., 2004).

Ideal process controls when extracting NoV and other enteric viruses from various matrices are genetically related viruses that should fulfill several criteria such as the ability to be cultivated easily using a cell culture and a similarity to the target virus. Moreover, it should be unlikely that it could naturally contaminate the tested food or clinical sample.

The murine norovirus 1 (MNV-1), the feline calicivirus (FCV), a genetically modified mengovirus (vMC_0) and MS2 bacteriophage have been most described as process control when detecting NoV and other enteric viruses in food samples, besides less used alternatives such as the canine calicivirus (CaCV) and poliovirus (PV).

vMC_0, a genetically modified mengovirus strain has been used widely as process control for detection of enteric viruses. Since the *Mengovirus* genus is a member of the *Picornaviridae* family, it is more related to the hepatitis A virus than to NoV. This modified strain is not pathogenic to humans and can be cultivated in HeLa-cells (Hahn and Palmenberg, 1995). It has been used as process control for detection of NoV and HAV, mostly in shellfish. A study by Le Guyader et al. (2009) described a recovery of 16 to 32 % in bio-accumulated oyster digestive tissue, which was 2 to 16 fold higher compared to GI and GII NoV recoveries (Le Guyader et al., 2009). Other studies analyzing the use of vMC_0 as process control in bio-accumulated oysters and mussels reported lower recovery efficiencies ranging between 0.2 % (Comelli et al., 2008) and 1.2-1.8 % (Uhrbrand et al., 2010), while NoV recoveries were similar to these found in the study by Le Guyader et al. (2009). A 1 % recovery of the vMC_0 process control when detecting HAV in bottled water was considered necessary to obtain correct results regarding HAV recovery from this matrix (da Silva et al., 2007). The use of vMC_0 has been suggested by the CEN/TC275/WG6/TAG4 working group as a process control for detection of enteric viruses in produce, shellfish and bottled water.

Although FCV is also a member of the *Caliciviridae* family, it belongs to the *Vesivirus* genus. It is highly infectious to cats and causes an oral and upper respiratory tract disease, but is not infectious to humans (Radford et al., 2007). Cultivation of this virus is possible in a feline kidney cell line (Bidawid et al., 2003). This virus has been used as process control when detecting NoV in shellfish and a good correlation between recoveries of GII.7 and FCV has been observed (Uhrbrand et al., 2010). Kingsley et al. (2007) showed successful recovery of 5.6 FCV RT-PCRU from 25g of inoculated oyster digestive tissue. Recovery of 250 plaque forming units (PFU) in 250 ml bottled water was possible (Mattison et al., 2009a), and a 34 % recovery has been observed when inoculating 66 FCV median tissue culture infective dose ($TCID_{50}$) in 1.5 l of the same matrix (Schultz et al., 2010). In the latter study, GII NoV were recovered with a similar efficiency, but GI NoV were recovered more efficiently.

MNV-1 is a genogroup V norovirus and thus belongs to the same genus (*Norovirus*) and family (*Caliciviridae*) as human infective NoV. Only mice can be infected with MNV-1, and clinical symptoms can vary according to the induced immunodeficiency (Karst et al., 2003). Cultivation is possible using the mouse macrophage cell line (RAW264.7 cells)(Wobus et al., 2006). Its use as process control when detecting human infective NoV has been proposed due to its genetic similarities towards the NoV genome. The use of MNV-1 as process control when detecting NoV (and other enteric viruses) in foods has been limited yet. MNV-1 has been successfully tested as process control in shellfish, as recovery of 14.5 MNV-1 RT-PCRU was possible in 25g of bio-accumulated oysters (Kingsley, 2007).

The bacteriophage MS2 has also been proposed as a process control for detection of NoV in foods and water (Blaise-Boisseau et al., 2010). MS2 is a nonpathogenic levivirus of the *Leviviridae* family and can be cultivated using the E. coli K-12 (ATCC12345) bacterial strain (Dawson et al., 2005). A good correlation has been noticed between the recovery of HAV and MS2 in bottled water, tap water, fresh and frozen strawberries. A 10 % recovery of the process control when detecting HAV in bottled water was considered necessary to obtain correct results regarding HAV recovery from these matrices (Blaise-Boisseau et al., 2010).

Reverse transcription controls (RTC)

The reverse transcription reaction is in particular prone to inhibition when detecting viral agents in shellfish (Loisy et al., 2005; Milne et al., 2007; Schwab et al., 1998), water (Laverick et al., 2004) and other foods (Love et al., 2008). Therefore, several control systems have been described whereby exogenous RNA is added to the reverse transcription reaction mixes as RTC, often referred to as internal RNA control in scientific literature.

A first system consists of the inclusion of RNA molecules in a viral capsid and is called “armored RNA”. This system ensures (long-term) stability of RNA, and a number of commercial kits are available to create these armored RNAs (Beld et al., 2004; Hietala and Crossley, 2006). This system has been used for detection of NoV in shellfish (Greening and Hewitt, 2008) and clinical samples (Medici et al., 2008)

Another RTC system consist of *in vitro* transcribed ssRNA fragments containing the primer-probe binding sites of a subsequently used (real-time) PCR detection assay and has been used for detection of enteric viruses in shellfish and clinical samples (Costafreda et al., 2006; Escobar-Herrera et al., 2006; Schwab et al., 1997; Trujillo et al., 2006a). An advantage of this method is the availability of commercial kits for the *in vitro* transcription of the RNA molecules. A problem with these first two systems is the relative instability of RNA, especially in case of long-term storage (Stevenson et al., 2008). Therefore, a third type of RTC makes use of RNA extracted from cultivable surrogate viruses such as the MS2 bacteriophage

which allows a constant availability of fresh RNA, although handling time may be increased due to need for cultivation (Dreier et al., 2005).

PCR controls

PCR inhibition – whether or not in real-time format – is a thoroughly described phenomenon when detecting pathogens in foods (Rijpens and Herman, 2002), water (Guy et al., 2003), shellfish (Abolmaaty et al., 2007) and clinical samples (Monteiro et al., 1997; Oikarinen et al., 2009) and many authors have suggested the use of a PCR internal amplification (IAC) control to detect possible false-negative results related to this inhibition (Hoorfar et al., 2004; Reiss and Rutz, 1999; Scipioni et al., 2008a). Moreover, the European Standardization Committee (CEN), in collaboration with International Standard Organization (ISO) has proposed a general guideline for PCR testing of food-borne pathogens that requires presence of IAC in the reaction mixture (Anonymous, 2002). In essence, an IAC consists of the addition of exogenous DNA to the PCR reaction mix.

A first approach to control PCR is the use of competitive PCR IAC, wherein the same primers (and probes) are used for amplification of IAC and target in the same tube. Advantages of this approach are (1) the ease of use as no extra PCR assay has to be designed and (2) a more correct accurate reflection of the (inhibition of the) amplification of the target sequence. The main disadvantage is the potential for reduced amplification efficiency of the target sequence due to a competition shift in favor of the IAC. The risk of the latter could be reduced (although not eliminated) by assuring that the amplicon size of the IAC exceeds that of the target. This type of IAC has been used for detection of NoV in water (Parshionikar et al., 2004) and enteroviruses in environmental samples (Gregory et al., 2006)

A second approach to control PCR consists of the use of a non-competitive IAC, whereby different primers (and probes) are used for target and IAC in the same tube. In contrast to the competitive IAC, the use of non-competitive IAC may not accurately reflect amplification of the target sequence which emphasizes the use of very similar target sequences for the IAC. Although reduced, competition for PCR components may still occur in case of a non-competitive IAC. Therefore, the concentration of the used IAC (whether or not competitive) should be kept as low as possible to avoid false-negative results (Hoorfar et al., 2003; Hoorfar et al., 2004). This system has been used for detection of NoV in faecal sample (Scipioni et al., 2008a).

1.3.8.3. Negative controls and contamination prevention

Negative controls are needed when detecting pathogens using molecular methods, as even the slightest contamination can lead to false-positive results, especially when very sensitive molecular methods are used (Borst et al., 2004; Niesters, 2002). This contamination can

result from sample-to-sample contamination as well as from the carryover of DNA from a previous amplification of the same target (Rijpens and Herman, 2002). A recent study investigating the presence of NoV in bottled water highlighted the importance of negative controls by showing that the majority of positive results were due to cross-contamination (Lamothe et al., 2003). To avoid false positive results from sample-to-sample contamination, a constant need remains to respect dedicated environmental conditions (separate working areas, UV decontamination, dedicated pipettes) if real-time PCR or conventional PCR are applied (Kwok and Higuchi, 1989). All reagents used in PCR must be prepared, divided into aliquots, and stored in an area free of PCR-amplified product, while addition of template DNA/RNA should occur in a another separated area. To reduce carryover contamination, Longo et al. (1990) described the use of uracil DNA-glycosilase (UNG) in combination with deoxyuridine triphosphate (dUTP) rather than deoxythymidine triphosphates (dTTP). UNG catalyzes the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP (during the initial denaturation phase of PCR). Additional contamination preventing measures include the sterilization of the working area by shortwave UV irradiation (Cimino et al., 1990; Cimino et al., 1991) as well as the use of aerosol-tight pipettes and sterile plastic disposables and glassware (Rijpens and Herman, 2002). It is also important to mention that high concentrated sodium hypochlorite solutions effectively destroy both nucleic acids and viral/bacterial pathogens, while ethanol based disinfectants – while very effective against bacterial pathogens – have limited disinfecting effect on viruses and do not destroy nucleic acids. It has been stated that the risk of DNA contamination has decreased by real-time PCR, due to the closed system which avoids the necessity for the post-PCR handling of amplified material (Klein, 2002; Mackay et al., 2002).

1.4. Conclusions

In summary, NoV are generally considered as a major and global cause of food borne gastroenteritis with various transmission routes. Its widespread presence is facilitated by features such as a low infectious dose and a high environmental resistance. Detection of NoV from foods differs at several points from detection of food borne bacteria and remains a challenge despite recent advances. For the molecular detection step, RT-PCR is now the accepted standard method besides alternatives such as NASBA and RT-LAMP and several sensitive RT-PCR assays are available. Nevertheless, advances such as multiplex assays and the use of PCR controls could improve the performance and reliability of these assays and assist the translation towards routine use. On the other hand, a wide variety of methods is available for the virus extraction step, which can be considered a bottleneck due to lower recovery efficiencies and the time-consuming protocols. A thorough evaluation of these virus extraction methods could help to solve these difficulties.

Chapter 2

Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus1

Redrafted after

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2. CHAPTER 2: MULTIPLEX REAL-TIME RT-PCR FOR SIMULTANEOUS DETECTION OF GI/GII NOROVIRUSES AND MURINE NOROVIRUS 1.

2.1. Abstract

A quantitative two-step multiplex real-time reverse transcriptase (RT-) PCR assay for the simultaneous detection of genogroup I (GI) and genogroup II (GII) noroviruses (NoV) is described below. A murine norovirus 1 (MNV-1) real-time PCR detection assay described recently, was integrated successfully into the multiplex assay, making it possible to detect GI and GII NoV and MNV-1 in one reaction tube with MNV-1 plasmid DNA as real-time PCR internal amplification control (IAC).

The results showed a nearly complete concordance between the multiplex assay and the corresponding single-target PCRs. Analysis of competition between the individual reactions within the multiplex real-time PCR assay showed that GI and GII NoV plasmid DNAs mixed at equimolar concentrations were detected reproducibly and quantitatively, while a 4 log excess between GI and GII plasmid DNAs hindered amplification of the target with the lowest concentration. High concentrations of the real-time PCR IAC (MNV-1 plasmid DNA) also interfered with the the possibility of the developed multiplex real-time RT-PCR assay to detect quantitatively and simultaneously the presence of GI and GII NoV within one sample.

The specificity of the multiplex assay was evaluated by testing a NoV RNA reference panel containing nine GI, eight GII, and one GIV in vitro synthesized RNA fragment, plus 16 clinical samples found positive for GI and GII NoV previously. In addition, a collection of bovine NoV and other (non – NoV) enteric viruses were found to be negative, and no cross-amplification between genogroups was observed.

2.2. Introduction

Noroviruses (NoV) are recognized as the single most common cause of gastroenteritis in people of all age groups worldwide (Koopmans and Duizer, 2004). NoV infections result frequently from person-to-person transmission in cruise ships (Chimonas et al., 2008; Depoortere and Takkinen, 2006) and hospitals (Gallimore et al., 2008). Other causes of infection are ingestion of contaminated food (De Wit et al., 2007; Gallimore et al., 2005; Johansson et al., 2002) and water (Craun et al., 2005; Schvoerer et al., 1999). The *Norovirus* genus belongs to the *Caliciviridae* family and can be subdivided into five genogroups (GI, GII, GIII, GIV, and GV), of which GI, GII, and GIV NoV are infectious to humans (Koopmans et al., 2002). However, only a small number of outbreaks due to genogroup IV NoV has been reported (Fankhauser et al., 2002; Koopmans, 2008). Genogroup III consists of bovine NoV

(van der Poel et al., 2003) and genogroup V contains the MNV-1 murine norovirus 1 (Wobus et al., 2006).

There is no reliable culture method available to detect NoV (Duizer et al., 2004), although efforts have been made recently (Asanaka et al., 2005; Straub et al., 2007). Currently, (real-time) reverse transcriptase (RT-) PCR is considered to be the gold standard for detection of NoV in clinical, food and environmental samples (Baert et al., 2007; Jothikumar et al., 2005b; Park et al., 2008; Wolf et al., 2007). Recently, several (multiplex) real-time RT-PCR assays for detection of GI and GII NoV in clinical samples (Pang et al., 2005) and in different food matrices such as shellfish (De Medici et al., 2004; Jothikumar et al., 2005b) and raspberries (Le Guyader et al., 2004a) have been published. Although the use of an appropriate (real-time) PCR internal amplification control (IAC) is an absolute requirement to avoid false-negative results due to malfunction of the thermal cycler, incorrect PCR mixture, poor DNA polymerase activity or, importantly, the presence of inhibitory substances in the sample matrix (Hoorfar et al., 2004; Malorny et al., 2003; Niesters, 2002), only a few assays for real-time PCR detection of NoV have been reported where a (real-time) PCR IAC was included. These assays used two types of DNA: either DNA originating from NoV surrogates, such as the MS2 bacteriophage (Dreier et al., 2005; Rolfe et al., 2007) and a genetically modified cultivable mengovirus (vMC_0) (Comelli et al., 2008), or nucleotide fragments containing NoV-specific primer binding sites (Escobar-Herrera et al., 2006; Scipioni et al., 2008b). In this study, the use of MNV-1 plasmid DNA as real-time PCR IAC was based on the ease of cultivation and quantification of this virus (Wobus et al., 2006), making it also possible to use MNV-1 virus particles as process control when detecting NoV from clinical, food and environmental samples. In addition, recent studies demonstrated that MNV-1 virus particles behave more like human NoV than other frequently used NoV surrogates, such as MS2 bacteriophage, feline calicivirus and poliovirus (Bae and Schwab, 2008).

This study describes the optimization of a multiplex real-time RT-PCR assay for the detection of human GI and GII NoV with the successful integration of MNV-1 as real-time PCR IAC. This assay combined available primers and probes for the detection of the majority of the human infective GI and GII NoV strains, designed by the CEN/TC/WG6/TAG4 working group (Loisy et al., 2005; Svraka et al., 2007), and primers and probes for the detection MNV-1 (Baert et al., 2008b) in a multiplex real-time RT-PCR assay. MNV-1 plasmid DNA was used as PCR IAC when testing GI and GII NoV positive (clinical) samples.

2.3. Materials & Methods

2.3.1. Clinical specimens and Norovirus Reference Panel.

Five GI and eleven GII NoV samples were obtained from clinical specimens submitted to the Belgian Scientific Institute of Public Health (IPH; Brussels, Belgium) and the Rega Institute for Medical Research (Leuven, Belgium) during a 6-year period (2002-2008).

An RNA NoV reference panel designed by the Dutch National Institute for Public Health and the Environment (RIVM; Bilthoven, the Netherlands) was also tested in this study. The reference panel contained *in vitro* synthesized RNA fragments covering genomic regions A, B and C (Vinje et al., 2004) of nine GI, eight GII and one GIV NoV. In addition, seven RNA preparations from other enteric viruses (rotavirus, astrovirus types 1 and 4, sapovirus, feline calicivirus, canine calicivirus and hepatitis A virus), kindly provided by the RIVM, and cDNA from four bovine GIII NoV, kindly provided by Liège University (Ulg;(Mauroy et al., 2008)), were included in this study. An overview of all tested samples / RNA fragments is shown in Table 2.1.

2.3.2. Viral RNA extraction.

Viral RNA was extracted from 100 µl clinical samples (10 % diluted in PBS) by using the RNeasy Mini kit (Qiagen, Hilden, Germany), according to the RNA Cleanup protocol, or by using the Viral RNA mini kit (Qiagen) according to the manufacterer's instructions, then stored at -20°C.

2.3.3. Reverse transcription.

A pre-reaction mix consisting of extracted/ in vitro synthesized RNA and random hexamers (Applied Biosystems, Foster City, CA, USA), in a final volume of 11.5 µl, was heated to 95°C during 2 min, then cooled on ice during 2 min (thus avoiding the presence of secondary structures in the RNA and allowing the full hybridization of the RNA with the random hexamers). This first pre-reaction mix was then mixed with a second pre-reaction mix of 8.5 µl to obtain a final 20 µl RT-mastermix containing 2.5 µM random hexamers (Applied Biosystems), 25 U of Multiscribe reverse transcriptase (Applied Biosystems), 20 U of RNase inhibitor (Applied Biosystems), 5 mM $MgCl_2$ (Applied Biosystems), 1×PCR buffer II (10 mM Tris HCl pH 8.3, 50 mM KCl; Applied Biosystems), 0.1 mM dNTPs (GE Healthcare; Diegem, Belgium) and extracted/ in vitro synthesized RNA. Reverse transcription was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) with the following temperature profile: 22°C for 10 min, 42°C for 15 min, 99°C for 5 min and 5°C for 5 min. All copy DNA (cDNA) was stored at -20°C.

Table 2.1 Overview of all tested samples/RNA fragments.

Virus type / NoV genotype	Sample type	Source	Ct GI	Ct GII	Ct MNV-1
GI.?	Faeces	IPH[a]	38.46	Undet	27.89
GI.1 (Norwalk)	RNA fragment	RIVM[b]	29.01	Undet	Undet
GI.2 (Whiterose)	RNA fragment	RIVM	20.52	Undet	Undet
GI.2	Faeces	REGA[c]	29.79	Undet	27.70
GI.2	Faeces	REGA	28.07	Undet	27.69
GI.2 (Southhampton)	RNA fragment	RIVM	20.83	Undet	Undet
GI.3 (Birmingham)	RNA fragment	RIVM	19.09	Undet	Undet
GI.4 (Malta)	RNA fragment	RIVM	19.33	Undet	Undet
GI.4	VTM[d]	REGA	26.04	Undet	27.56
GI.5 (Musgrove)	RNA fragment	RIVM	39.12	Undet	Undet
GI.6 (Mikkeli)	RNA fragment	RIVM	19.62	Undet	Undet
GI.7 (Winchester)	RNA fragment	RIVM	17.54	Undet	Undet
GI.8	Faeces	REGA	22.76	Undet	27.32
GI.10 (Boxer)	RNA fragment	RIVM	19.34	Undet	Undet
GII.1 (Hawaii)	RNA fragment	RIVM	Undet	19.46	Undet
GII.2 (Melksham)	RNA fragment	RIVM	Undet	18.66	Undet
GII.2	Faeces	REGA	Undet	29.92	27.76
GII.3 (Toronto)	RNA fragment	RIVM	Undet	21.78	Undet
GII.4 (Grimsby)	RNA fragment	RIVM	Undet	18.26	Undet
GII.4	Vomit	REGA	Undet	28.95	27.83
GII.4	Faeces	REGA	Undet	22.90	27.82
GII.4	Faeces	REGA	Undet	21.63	28.78
GII.?	Faeces	IPH	Undet	28.92	27.79
GII.?	Faeces	IPH	Undet	26.30	27.41
GII.?	Faeces	IPH	Undet	33.57	27.89
GII.?	Faeces	IPH	Undet	25.72	27.37
GII.?	Faeces	IPH	Undet	26.28	27.61
GII.?	Faeces	IPH	Undet	27.05	27.58
GII.6 (Seacroft)	RNA fragment	RIVM	Undet	22.07	Undet
GII.7	Faeces	IPH	Undet	21.48	Undet
GII.10 (Erfurt)	RNA fragment	RIVM	Undet	18.49	Undet
GIIb (GGIIb)	RNA fragment	RIVM	Undet	19.05	Undet
GIIc (GGIIc)	RNA fragment	RIVM	Undet	19.21	Undet
GIV (Alphatron)	RNA fragment	RIVM	35.87	Undet	Undet
GIII (Bovine)	Faeces	Ulg[d]	Undet	Undet	Undet
GIII (Bovine)	Faeces	Ulg	Undet	Undet	Undet
GIII (Bovine)	Faeces	Ulg	Undet	Undet	Undet
GIII (Bovine)	Faeces	Ulg	Undet	Undet	Undet
Rotavirus	Faeces	RIVM	Undet	Undet	Undet
Astrovirus type 1	Faeces	RIVM	Undet	Undet	Undet
Astrovirus type 4	Faeces	RIVM	Undet	Undet	Undet
Sapovirus	Faeces	RIVM	Undet	Undet	Undet
Feline Calicivirus	Faeces	RIVM	Undet	Undet	Undet
Canine Calicivirus	Faeces	RIVM	Undet	Undet	Undet
Hepatitis A virus	Faeces	RIVM	Undet	Undet	Undet

[a]IPH: Belgian Scientific Institute of Public Health, [b]RIVM: Dutch National Institute for Public Health and the Environment, [c]REGA: Rega Institute for Medical Research, [d]Ulg: Liège University, [e] VTM: Viral transport medium.

2.3.4. Real-time PCR.

2.3.4.1. Generation of plasmid standards.

To obtain representative positive control standards, the previously described plasmid p20.3 was used for the quantification of MNV-1 (Baert et al., 2008b), while plasmids containing primers-probe binding sites were constructed for GI and GII NoV. For GI NoV, a 100 bp PCR amplicon (ATGCCATGTTCCGCTGGATGCGCTTCCATGACCTCGGATTGTGGACAGGAGATCGCGATCTTCTGCCCGAATTCGTAAATGATGATGGCGTCTAAGGAAT) covering the primers-probe binding sites (underlined) was cloned into the pMOS Blue vector (Amersham Biosciences, Saclay, France), resulting in the pGI plasmid. For GII NoV, a 102 bp PCR amplicon (TTCAAGAGTCAATGTTTAGGTGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGCTTTGTGAATGAAGATGGCGTCGATT) covering the primers-probe binding sites (underlined) was cloned into the pGEM-T-Easy vector (Promega, Leiden, the Netherlands), resulting in the pGII plasmid. Plasmid DNA was purified by using a Plasmid Midi Kit (Qiagen). The plasmid concentration was determined by photospectroscopy at 260 nm using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Ten-fold serial dilutions ranging from 10^7 to 10 copies of all three positive control plasmids were used to prepare the standard curves.

2.3.4.2. Primers and probes.

Primers and probes for the individual quantification of GI and GII NoV were designed by the CEN/TC/WG6/TAG4 working group (Loisy et al., 2005; Svraka et al., 2007). The primers and probe for the individual quantification of MNV-1 were designed by Baert et al. (2008b). An overview of the primers and probes sequences is shown in Table 2.2. All primers and probes were purchased from Eurogentec (Liège, Belgium), except the NED-labeled Minor Groove Binding (MGB) TaqMan probe, which was purchased from Applied Biosystems.

Table 2.2 Overview of primers and probes used for real-time RT-PCR

Primers/probes	Sequence (5' – 3')[a]	Polarity [b]	Position[c]	Final conc	Fluorophore[d] (5')/ Quencher (3')
NoV GI					
QNIF4	CGCTGGATGCGNTTCCAT	+	5291-5308	500 nM	
NV1LCR	CCTTAGACGCCATCATCATTTAC	-	5354-5376	900 nM	
NVGG1p	TGGACAGGAGAYCGCRATCT	+	5321-5340	100 nM	6-FAM/BHQ-1
NoV GII					
QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	+	5012-5038	500 nM	
COG2R	TCGACGCCATCTTCATTCACA	-	5100-5080	900 nM	
QNIFS	AGCACGTGGGAGGGCGATCG	+	5042-5061	250 nM	Texas Red/BHQ-1
MNV-1					
FW-ORF1/ORF2	CACGCCACCGATCTGTTCTG	+	4972-4991	200 nM	
RV-ORF1/ORF2	GCGCTGCGCCATCACTC	-	5064-5080	200nM	
MGB-ORF1/ORF2	CGCTTTGGAACAATG	+	5001-5015	200nM	NED/MGBNFQ

[a] Mixed bases in degenerate primers and probes are as follows: Y, C or T; R, A or G; N, any;
[b] +, virus sense; -, anti-virus sense
[c] Corresponding nucleotide position of Norwalk/68 virus (accession nr. **M87661**) for NoV GI, Lorsdale virus (accession nr. **X86557**) for NoV GII or murine norovirus 1 clone CW1 (accession nr. **DQ285629**).
[d] BHQ-1: Black Hole Quencher – 1, MGBNFQ: Minor Groove Binding Non-Fluorescent Quencher

2.3.4.3. Real-time PCR assay.

Quantitative real-time PCR was carried out in a 25 µl reaction mix containing 1 µl of template DNA and 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), which contains dUTP and uracyl N-glycosylase (UNG). Primers and probes were used in the concentrations given in Table 2.2. In some cases, 10^3 copies of plasmid p20.3 (Sosnovtsev et al., 2006) were added to this reaction mix as real-time PCR IAC. Real-time quantification was performed on the Lightcycler LC480 real-time PCR instrument (Roche Diagnostics, Mannheim, Germany) under the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation/activation at 95°C for 10 min, followed by 50 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Amplification data were collected and analyzed with the LC480 instruments' software. The amplification efficiency (E) was calculated from the plasmid standard curves using the equation $E=(10^{(-1/slope)} -1)\times100$. To minimize cross-talk between the different channels of the real-time PCR instrument, a minimal wavelength difference of 25 nm was taken between both excitation and emission maxima of the different fluorescent labels. Eventual cross-talk was minimized by applying the color compensation as described in the LC480 manual.

2.4. Results

2.4.1. Singleplex real-time PCR assays for GI and GII NoV and MNV-1.

Plasmids pGI, pGII and p20.3 – containing primers-probe binding sites of GI and GII NoV and MNV-1, respectively – were each 10-fold serially diluted in water and subjected to the singleplex real-time PCR assays. Fig. 2.1 shows the standard curves of these three

singleplex assays. Analysis of the parameters of the standard curves of replicates of two independent runs showed that the three singleplex assays (GI NoV, GII NoV and MNV-1) were sensitive (detection limits of 10 copies) and efficient (PCR efficiencies of 91.6 %, 87.3 % and 94.2 %, respectively). Standard deviations were small (with a maximum of 0.92 Ct), and the square regression coefficient (R^2) value was ≥ 0.998 for all three singleplex assays in the concentration range tested. Lastly, all intercepts were within a 2.9 Ct range.

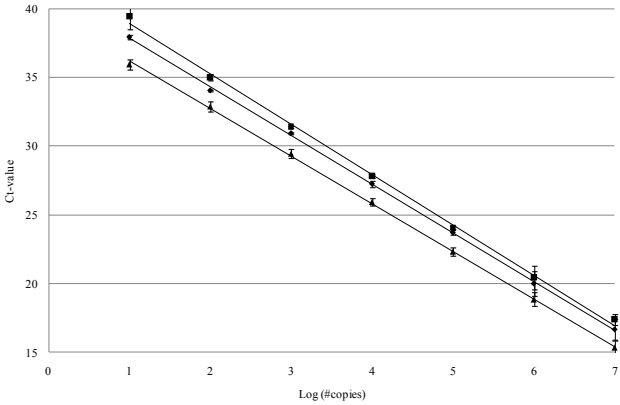

Fig. 2.1: Standard curves for the three singleplex GI, GII and MNV-1 real-time PCR detection assays using 10-fold serially diluted plasmid standards of pGI (series ◆) , pGII (series ■) and p20.3 (series ▲), respectively, ranging from 10^7 to 10 copies.

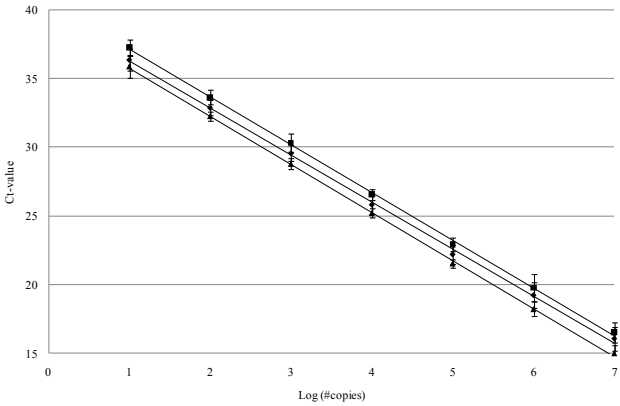

Fig. 2.1: Standard curves for the three individual GI, GII and MNV-1 real-time PCR detection reactions within the multiplex real-time RT-PCR detection assays using 10-fold serially diluted plasmid standards of pGI (series ◆) , pGII (series ■) and p20.3 (series ▲), respectively, ranging from 10^7 to 10 copies.

2.4.2. Multiplex real-time PCR for detection of GI/GII NoV and MNV-1.

To examine the possible competition between the three individual PCR reactions within the multiplex real-time assay, plasmids pGI, pGII and p20.3 were mixed in equimolar amounts ranging from 10^7 to 10 copies. A comparison of parameters of the standard curves of duplicates of five independent multiplex runs (shown in Fig. 2.2) with those of the singleplex runs (shown in Fig. 2.1) showed that Ct values are in accordance with each other, with a maximum difference of less than one Ct. Furthermore, all parameters of the standard curves of the individual GI, GII and MNV-1 reactions within the multiplex PCR indicated that these individual reactions were sensitive (detection limit of 10 copies) and efficient (PCR efficiencies of 96.1 %, 93.8 % and 93.1 %). Again, standard deviations were small (with a maximum of less than 1 Ct), and the R^2-value was ≥ 0.999 for all three individual PCRs within the multiplex assay in the concentration range tested. Lastly, all intercepts were within a 1.5 Ct range. These data suggest that reliable quantitative detection of the GI/GII NoV and MNV-1 within the same sample is possible on the LC480 instrument using the multiplex real-time PCR assay.

The competitive effect between the individual PCR reactions within the multiplex assay was tested further by preparing all possible combinations of quantities of 0, 10, 10^3 and 10^5 copies of pGI, pGII and p20.3 and submitting these combinations to the multiplex assay. The resulting Ct values are shown in Fig. 3.1. In particular, this analysis focused on competitive effects between the NoV GI and GII reactions (to analyze the possibility of detecting both GI and GII NoV within one sample), as well as the competition between the MNV-1 reaction and both the GI and GII reactions (to analyze the possibility of using MNV-1 as real-time PCR IAC), all within the multiplex assay.

The effect of the presence of GII on the GI reaction within the multiplex assay was not negligible (Fig. 2.3 A-B-C). 10^5 copies of plasmid pGI were detected at the expected Ct value in the presence of 10, 10^3 and 10^5 copies of pGII (Fig 3A). 10^3 copies of pGI were detected at the expected Ct value in the presence of 10 or 10^3 copies of pGII, while a 2.8 Ct increase was observed in the presence of 10^5 copies of pGII (Fig. 2.3B). Ten copies of pGI were detected at the expected Ct value in the presence of 10 copies of pGII. However, a 2.9 Ct-shift was noticeable in the presence of 10^3 copies of pGII while these ten copies of pGI could not be detected (Ct>50) in the presence of 10^5 copies of pGII (Fig 2.3C).

Similarly, the presence of GI affected the GII reaction within the multiplex assay when high amounts (10^5 and 10^3 copies) of pGII were combined with any copy number (0, 10, 10^3 and 10^5 copies) of pGI (Fig. 2.3D, 2.3E). Similarly, a 2.2 Ct-shift was noticeable when 10^3 copies of pGII were detected in the presence of 10^5 copies of pGI (Fig. 2.3E). Ten copies of pGII were detected as expected in the presence of 10 copies of pGI. However, a 1.8 Ct-shift was

noticeable in the presence of 10^3 copies of pGI while ten copies of pGII could not be detected (Ct>50) in the presence of 10^5 copies of pGI (Fig. 2.3F).

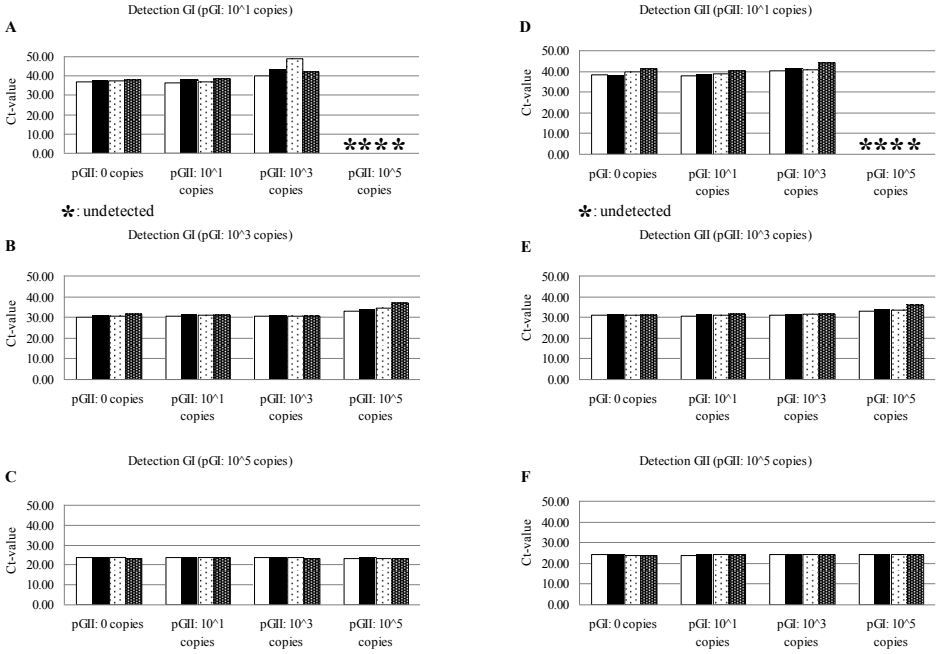

Figure 2.3: **A-B-C**: The effect of the presence of GII on Ct values (vertical axis) of the GI reaction within the multiplex real-time PCR assay. Different copy numbers (0, 10, 10^3 and 10^5 copies) of pGII (horizontal axis) are combined with 10 (Fig 3A), 10^3 (Fig 3B) and 10^5 (Fig 3C) copies of pGI. **D-E-F**: The effect of the presence of GI on Ct values (vertical axis) of the GII reaction within the multiplex real-time PCR assay. Different copy numbers (0, 10, 10^3 and 10^5 copies) of pGI (horizontal axis) are combined with 10 (Fig 3A), 10^3 (Fig 3B) and 10^5 (Fig 3C) copies of pGII. The effect of the presence of MNV-1 on the GI and GII reactions within the multiplex real-time PCR assay was also included in figure 3. Copy numbers of 0 (series □), 10 (series ■), 10^3 (series □) and 10^5 (series ▩) of p20.3 were combined with any combination of copy numbers of pGI and pGII. All Ct values are means of duplicates.

Overall, the effect of the MNV-1 reaction on the GI and GII reactions within the multiplex assay was limited when pGI or pGII were solitarily present, as only a 4 log excess (10^5 copies) of plasmid p20.3 over pGI or pGII (10 copies) caused a Ct-shift ranging from 0.9 to 2.7 Cts (Fig. 2.3A, 2.3D). On the other hand, the effect of the MNV-1 reaction on the GI and GII reactions within the multiplex assay was not negligible when pGI and pGII were both present in one sample. When 10 and 10^3 copies of pGI were combined with 10^3 and 10^5 copies of pGII, respectively, Ct-shifts ranging from 2.1 to 8.9 and 1.6 to 3.8, respectively, were caused by the presence of 10^3 or 10^5 copies of p20.3. Similarly, when 10 and 10^3

copies of pGII were combined with 10^3 and 10^5 copies of pGI, respectively, Ct-shifts ranging from 0.5 to 3.8 and 0.2 to 3.8, respectively, were caused by the presence of 10^3 or 10^5 copies of p20.3.

2.4.3. Analysis of the specificity of the multiplex real-time RT-PCR.

The specificity of the multiplex assay was analyzed by subjecting a Norovirus RNA Reference Panel containing in vitro synthesized RNA fragments covering genomic regions A, B and C (Vinje et al., 2004) of nine GI, eight GII and one GIV NoV and 16 clinical GI/GII NoV samples to this assay (Table 2.1). All tested genotypes in the Norovirus RNA reference panel were detected specifically, all clinical samples found positive for GI (5 samples) or GII (11 samples) NoV previously were confirmed and no cross-amplification between the different GI, GII and GIV genotypes was observed. The seven alternative virus strains and the bovine GIII NoV were not detected. 10^3 copies of the p20.3 plasmid used as real-time PCR internal amplification control were detected at expected Ct value (~28), suggesting that no PCR inhibitory components were present in the cDNA preparations of the clinical samples.

2.5. Discussion

The current study describes the successful combination of three singleplex real-time PCR assays for detection of GI/GII NoV and MNV-1 into one multiplex real-time RT-PCR assay. Primers and probes in all three singleplex assays target the ORF1-ORF2 junction regions (ORF: open reading frame), which are considered to be the most conserved region of the NoV genome (Kageyama et al., 2003; Nishida et al., 2003).

All singleplex PCR reactions proved to be sensitive, with detection limits of 10 copies of the pGI, pGII and p20.3 plasmids (containing the primers-probe binding sites of GI/GII NoV and MNV-1, respectively). Other authors reported similar detection limits ranging between 1 and 10 genomic copies (Jothikumar et al., 2005b; Pang et al., 2005; Wolf et al., 2007). This low detection limit is necessary (1) because of the low viral concentration in environmental and food samples and (2) because of the low infectious dose of NoV; it is reported that only 10 virions could be enough to infect a healthy adult (Hutson et al., 2004; Teunis et al., 2008).

The combination of these three singleplex reactions into a multiplex assay requires similar PCR kinetics (Molenkamp et al., 2007; Persson et al., 2005). PCR-efficiencies of all singleplex assays were within a 9% range and intercepts differed less than 2.9 Cts.

When equally mixed amounts of the pGI, pGII and p20.3 plasmids were detected with the multiplex assay, only a negligible loss in sensitivity was observed in comparison to the singleplex reactions.

When pGI, pGII and p20.3 plasmids were mixed in different concentrations, a mutual competitive effect was noticeable between the individual GI and GII NoV reactions within the

multiplex assay. This competitive effect became clear when a 2 log concentration difference (10^5 / 10^3 copies and 10^3 / 10 copies) was present between the two targets (pGI and pGII), resulting in Ct-shifts between 1.8 and 2.9 Cts for the target present in the lowest concentration. Additionally, when a 4 log concentration difference (10^5 / 10 copies) was present between the 2 targets (pGI/pGII), the target with the lowest concentration could not be detected (Ct>50).

The effect of the MNV-1 reaction on the GI and GII NoV reactions within the multiplex assay was limited when pGI or pGII were solitarily present. However, the presence of 10^3 and 10^5 copies of p20.3 did cause additional Ct-shifts when both pGI and pGII were present in one sample.

This analysis showed the limits of the multiplex assay for the detection of low amounts of one NoV genotype (GI/GII) in the presence of high amounts of another NoV genotype (GII/GI) in the same sample. These results also indicated that the use of the MNV-1 reaction as real-time PCR internal amplification control (IAC) is achievable. To avoid (1) competitive effects and (2) the loss of the quantitative properties of the multiplex assay (especially when detecting low virus concentrations), no more than 10^2 to 10^3 copies of plasmid p20.3 should be added to the real-time PCR reaction as real-time PCR IAC when detecting GI/GII NoV.

A previous study of competitive effects between individual reactions within a multiplex PCR assay designed to simultaneously detect 4 virus types did not report analogous Ct-shifts (Molenkamp et al., 2007), but in this study only a 3-log difference between the target DNAs was investigated. However, the results of the current experiments support another multiplex real-time RT-PCR study (Candotti et al., 2004), in which Ct-delays (2 to 3 Cts) were reported when low concentrations of viral genome (50 to 10^3 genomic RNA copies) were detected simultaneously with another abundant viral genome (10^4 to 10^6 genomic RNA copies). Competition of individual PCR reactions within a multiplex (real-time) PCR is a known problem (Cook et al., 2002) and the results of the current study show that this issue should not be neglected during the design and optimization of quantitative multiplex real-time PCR assays, especially when detecting low-concentration DNA targets. Nevertheless, a well-optimized multiplex (real-time) PCR assay has benefits, including reduced expense of reagents and preparation time, combined with the possibility to include a (real-time) PCR IAC (Edwards and Gibbs, 1994).

Specificity of the multiplex assay was analyzed by testing a wide range of human GI, GII and GIV NoV genotypes, bovine GIII NoV genotypes and alternative virus strains. Specific detection of human NoV genotypes by real-time RT-PCR has been demonstrated before in other studies (Menton et al., 2007; Wolf et al., 2007). However, the Alphatron (GIV) NoV genotype was only included in a limited number of studies (Jothikumar et al., 2005b; Reuter et al., 2005; Trujillo et al., 2006b)) and GIIc NoV genotypes.

Multiplex real-time RT-PCR assays for the simultaneous detection of GI and GII NoV have been reported before (Jothikumar et al., 2005b; Kageyama et al., 2003; Pang et al., 2005) and many authors have suggested the use of a PCR IAC to detect possible false-negative results due to inhibition when detecting genomic material (Hoorfar et al., 2004; Reiss and Rutz, 1999; Scipioni et al., 2008b). This inhibition of (real-time) PCR assays is a known problem when detecting pathogens in faecal samples (Lantz et al., 1997; Monteiro et al., 1997; Oikarinen et al., 2009), sewage samples (Guy et al., 2003) and food matrices (Rijpens and Herman, 2002). Therefore, a cultivable MS2 bacteriophage (Dreier et al., 2005; Rolfe et al., 2007), a genetically modified cultivable mengovirus (Comelli et al., 2008) and a cDNA fragment (whether or not NoV-related) flanked by primer binding sites (Escobar-Herrera et al., 2006; Scipioni et al., 2008b) have recently been used as (multiplexed) PCR IAC. The use of DNA originating from a cultivable surrogate as (multiplexed real-time) PCR IAC is favored above the use of a cDNA fragment flanked by primer binding sites, as these cultivable surrogate organisms can also be utilized as (quantifiable) process control for the full extraction procedure when detecting GI and GII NoV in clinical, environmental and food samples.

A comparison between several cultivable NoV surrogates for the detection of human NoV in water favored the use of MNV-1 compared to other candidates such as MS2 bacteriophage, feline calicivirus (Bidawid et al., 2003) and poliovirus (Bae and Schwab, 2008). In addition, the similar biological properties of MNV-1 and human GI and GII NoV (Cannon et al., 2006; Wobus et al., 2006) make it a preferred process control in human NoV detection assays. Therefore, 10^3 copies of plasmid p20.3 (containing a full length MNV-1 cDNA genome) were used as real-time PCR IAC in the multiplex assay developed. Detection of this PCR IAC at expected Ct values suggested absence of PCR inhibitory compounds in the cDNA preparations of the tested clinical samples.

2.6. Conclusions

This chapter described the design of a multiplex real-time RT-PCR assay for detection of human GI and GII NoV in clinical samples, with the successful inclusion of MNV-1 as real-time PCR IAC. This multiplex real-time PCR assay can be used as a rapid method for detection of NoV in environmental and food samples as well, although the robustness of this assay should be further examined for these sample categories. Furthermore, this multiplex assay has been used throughout the chapters 4 to 6, in combination with different virus extraction and RNA purification methods.

2.7. Acknowledgements

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Chapter 3

LABORATORY EFFORTS TO ELIMINATE CONTAMINATION PROBLEMS IN THE REAL-TIME RT-PCR DETECTION OF NOROVIRUSES

Redrafted after

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3. CHAPTER 3: LABORATORY EFFORTS TO ELIMINATE CONTAMINATION PROBLEMS IN THE REAL-TIME RT-PCR DETECTION OF NOROVIRUSES.

3.1. Abstract

In the current study, laboratory efforts to prevent the presence of positive NTCs (no template controls) during the optimization of a quantitative real-time reverse transcriptase (RT-) PCR assay for detection of Noroviruses (NoV) are described. Two DNA types (single-stranded (ss)DNA fragments and plasmid DNA) were used to generate a real-time PCR standard and a high frequency of positive NTCs was noticed in the case of ssDNA fragments. To investigate our suspicion of well-to-well migration of DNA during real-time PCR runs as possible cause of the positive NTCs, an “evaporation-experiment” was set up in which the evaporation of water and the possible co-evaporation of DNA were measured as a function of the DNA type (ssDNA-fragments, plasmid DNA and genomic DNA), the reaction plate seal type (adhesive film or 8-cap strips) and the use of 7µl of mineral oil as cover layer. Results of this experiment indicated that evaporation of water occurred during real-time PCR runs regardless of the DNA type, the seal type and whether or not 7 µl of mineral oil was used as cover layer. Data from this experiment also suggested co-evaporation of DNA, with an apparent negative correlation between the size of the DNA type and the extent of this co-evaporation. The use of 7 µl of mineral oil as cover layer seemed to prevent to some extent co-evaporation of DNA. The use of plasmids as standard combined with 7 µl of mineral oil as cover layer in the real-time PCR setup resulted in a complete absence of positive NTCs while only minor effects were noticed on the performance of the real-time PCR. In general, our results showed that the high sensitivity of an optimized real-time PCR assay should be considered as – besides a great advantage – a potential risk factor for obtaining false-positive results when using this technique.

3.2. Introduction

PCR has become an established method for detection of food borne bacterial (Abubakar et al., 2007) and viral agents (Love et al., 2008; Rutjes et al., 2006a; Wolf et al., 2007). It is being increasingly used in surveillance studies and end product testing for detection of pathogens as it provides a rapid and sensitive tool for the screening of large numbers of clinical and environmental samples (Lampel et al., 2000). Since cultivation of human NoV strains require a complex cell system to grow (Asanaka et al., 2005; Straub et al., 2007), for now (real-time) reverse transcriptase PCR is considered as the gold standard for detection of NoV (Houde et al., 2006). The introduction of real-time PCR, the technological improvement

of PCR machines and the use of optimized buffers and enzymes greatly increased the PCR sensitivity. If optimized well, real-time PCR assays have the possibility to detect less than 10 copies, corresponding often to cycle threshold (Ct) values of 36-40 (Klein, 2002; Peters et al., 2004; Reynisson et al., 2006). A drawback of (real-time) PCR is that it is prone to contamination, leading to false-positive results (Borst et al., 2004; Niesters, 2002). This is particularly important when detecting NoV as there is no possibility yet to confirm positive PCR test results by culture (in contradiction to most bacterial pathogens). False-positives can result from sample-to-sample contamination and from carryover DNA originating from previous amplification of the same target (Speers, 2006). The introduction of real-time PCR combined with the use of enzymatic systems (Uracil N-Glycosidase (UNG)) has to a great extent dealt with the latter (carryover-) contamination issue (Kleiboeker, 2005; Pang et al., 1992). To avoid false positive results from sample-to-sample contamination, a constant need remains to respect dedicated environmental conditions (separate working areas, UV decontamination, dedicated pipettes, mineral oil, no template controls) if real-time PCR and conventional PCR are applied in the microbiological lab (Borst et al., 2004; Kwok and Higuchi, 1989; Rijpens and Herman, 2002).

Positive NTCs (no template controls) are a frequent observation in many labs when setting up or optimizing PCR protocols or executing PCR testing on a routine basis. Especially when manipulating high concentrations of target DNA in setting up real-time PCR standard curves or as positive control templates the risk of false-positive results increases (Espy et al., 2006). Although no guidelines have been published on this matter, attempts to interpret the occurrence of positive NTCs have been made (Bustin and Nolan, 2004). When amplification occurs in NTCs, high Ct values are often noticed, indicating contamination of only few copies of DNA in the NTC.

In the present chapter, the hypothesis was raised if the occurrence of positive NTCs could be due to evaporation of water during the PCR run also enabling co-evaporation of templating DNA and thus transfer to NTC's providing occasionally high Ct values for the NTC reactions.

3.3. Materials & Methods

3.3.1. Quantitative real-time PCR.

The real-time quantitative PCR was carried out in a volume of 25 µl. The reaction mix contained 5 µl of target DNA, 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) containing dUTP and UNG, 500 nM of the QNIF2-forward primer (5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3'; Eurogentec, Liège, Belgium), 900 nM of the COG2R-reverse primer (5'-TCGACGCCATCTTCATTCACA-3'; Eurogentec) and 250 nM of the QNIFS TaqMan-probe for Norovirus GGII detection (YakimaYellow-5'-AGCACGTGGGAGGGCGATCG-3'-BHQ1; Eurogentec). The QNIF2 and COG2R primers

and the QNIFS probe were designed by the CEN/TC275/WG6/TAG4 working group and ordered at Eurogentec (Liège, Belgium). PCR amplification was performed with a ABI Prism® 7000 Sequence Detection System (Applied Biosystems) under the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, followed by 50 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min.

Ten-fold serial dilutions of both (1) a 102 nucleotide synthetic ssDNA fragment "ssGII" (Eurogentec, Liège, Belgium) based on the NoV GII real-time RT-PCR protocol designed by Jothikumar et al (2005b) and (2) the "pGII" plasmid (size: 3117 bp) being a pGEM-T-easy vector (Promega, Wisconsin, USA) with an insert of 102 bp containing the primers-probe binding sites, served as standard positive controls in the real-time PCR (Table 3.1).

Table 3.1: Sequences of ssGII and insert of pGII. Primer and probe binding sites are underlined.

Positive control (length)	**Sequence (5' - 3')**
Synthetic ssDNA fragment "ssGII" (102 nucleotides)	TTCAAGAGTCATTGTTTAGGTGGATGAGATTCTCAGATCT GAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGCTT TGTGAATGAAGATGGCGTCGATT
Insert of "pGII" (102bp)	AGCTTTGTTCAGATGGATGAGATTCTCAGATCTGAGCACG TGGGAGGGCGATCGCAATCTGGCTCGGATCCAGCTTTGT GAATGAAGATGGCGTCGAAGCTT

When ssGII and pGII were used as template DNA, respectively a 89 bp and a 93 bp real-time PCR amplicon were generated. Sequence details are shown in Table 3.1. Negative template controls (NTCs) consisted of the real-time PCR reaction mix without DNA added, but instead with 5 µl of sterile HPLC-grade water. The number of NTCs per real-time PCR run varied between 4 and 18.

Both the MicroAmp™ Optical 8-Cap Strip and the MicroAmp™ Optical Adhesive Film (Applied Biosystems) were used as seal for the 96 well real-time PCR reaction plate (Applied Biosystems). In some cases, 7 or 30 µl of mineral oil (Sigma-Aldrich, St. Louis, USA) was used as cover layer on top of the 25 µl real-time PCR reaction mixtures.

Amplification data were collected and analysed with the ABI Prism® 7000 SDS software version 1.0 (Applied Biosystems). Sensitivity of the real-time PCR assay was analysed by evaluating Ct – values, while the reproducibility was examined on the basis of the square regression coefficient (R^2 – value) of the obtained real-time PCR standard curves.

To visualize and to measure the size of the real-time PCR amplicons present in the negative control (NTC) wells, agarose gel electrophoration was performed for 30 min at 100 V on a 4% (w/v) NuSieve® 3:1 Agarose (Lonza, Verviers, Belgium) in 0.5 × TAE buffer containing

400 mM Tris-acetate and 10 mM EDTA (Invitrogen Ltd., Paisley, UK). As a size marker, the 1 kb DNA ladder (Invitrogen Ltd.) was used. The agarose gels were visualised after staining with ethidium bromide (2 µg/ml), and photographed on a UV transillumination table with a Polaroid MP4 Land Camera (Polaroid Corp., Cambridge, MA, USA) using type 667 film.

3.3.2. Evaporation experiment.

3.3.2.1. Different DNA types.

A 104 nucleotide ssDNA-fragment (5'-TTGCACCACACAGCTGAATAGTTTGGCTCACTGGA TTTTGACCCTTTGTGCAATGGTTGAGGTAACCCGAGTTGACCCTGACATTGTGATGCAA GAATCTGATT-3') was purchased at Eurogentec, a pGEM-T-easy vector (Promega) containing an 81 bp insert (5'-TGATGCGATTCCATGACGATTGTGGGACAGAGATCGCGAT CTTCTGCGGATCCGAATTCGTGAAATGATGATGGCGTCTAA-3') and with a total plasmid size of ~3,1 kb was isolated by the alkaline lysis method of Birnboim and Doly (1979) and genomic DNA (size: ~4,6 mb) was extracted from DH5α *Escherichia coli* cells grown overnight at 37°C in Luria Broth Base medium (GIBCO BRL, Eggenstein, Germany) by the method of Flamm et al (1984). The three DNA types (ssDNA, plasmid DNA and genomic DNA) were diluted in sterile HPLC-grade water to a final concentration of approximately 200 ng/µl. The precise concentration of the DNA was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.3.2.2. Plate setup

Two 96-well reaction plates were prepared with identical setups: 25 µl solutions of each of the 3 DNA types (ssDNA fragments, plasmid DNA and genomic DNA) at the respective final concentrations (178.92, 281.94 and 236.00 ng/µl) were pipetted into respectively 12 different wells of each plate. No real-time PCR components were added to any of the solutions. Wells 1 to 12 contained ssDNA fragments, wells 13 to 24 contained plasmid DNA and wells 25 to 36 contained genomic DNA.

Seven µl of mineral oil (Sigma-Aldrich, St. Louis, Missouri) was used as cover layer on the DNA solutions in wells 1 to 3, 7 to 9, 13 to 15, 19 to 21, 25 to 27 and 31 to 33.

Moreover, each of the three DNA types was combined with two different seal types: the MicroAmp™ Optical 8-Cap Strip and the MicroAmp™ Optical Adhesive Film (Applied Biosystems). Wells 1 to 6, 13 to 18 and 25 to 30 were closed by the 8-cap strip, while wells 7 to 12, 19 to 24 and 31 to 36 were closed by the adhesive film. In summary, three replicates were taken for each of the 12 combinations (DNA type – with or without 7 µl of mineral oil – seal type).

One of the plates underwent a single real-time PCR thermal cycling program as described above, the other plate underwent 5 identical subsequent real-time PCR thermal cycling programs.

3.3.2.3. Statistics

All statistical analyses were done using the Statistica 8.0 software (StatSoft, Tulsa, OK, USA). Data from the "evaporation"-experiment were analysed using a two-factor analysis of variance (ANOVA), with factor one the DNA type and factor two the combination of seal type and eventual use of 7 µl mineral oil as cover layer.

3.4. Results

A comparison between the use of a ssDNA fragment (ssGII) and plasmid DNA (pGII), both containing the primer-probe binding sites, as standard in the quantitative real-time PCR showed that both assays were sensitive (detection limits of 10 copies with intercepts of 42.79 (ssGII) and 43.92 (pGII)), reproducible (R^2-value ≥ 0.99) and efficient (slope = -3.13 (ssGII) or -3.36 (pGII), corresponding to PCR efficiencies of 108.7 % and 98.5 % respectively).

Although the parameters of both standard curves showed that with both DNA types reliable standard curves were obtained, amplification was noticed in all 10 NTCs in 3 independent real-time PCR runs with ssGII as target DNA, with Ct-values ranging between 38.15 and 40.32, corresponding to an initial presence of about 10 copies of the target DNA. No amplification occurred in any of the NTCs when pGII was used as standard positive control. Agarose gel electrophoresis of the real-time PCR products of the NTCs showed the presence of a DNA-fragment with the same size of the amplicon in the positive controls (data not shown).

To avoid future problems, the cause of these positive NTCs was investigated. This investigation included a study of the possibility of well-to-well transfer of target DNA during the real-time PCR run as possible cause and the influence of both the reaction plate seal type and the eventual use of mineral oil as vapour barrier on the presence of the positive NTCs.

The effect of 3 DNA types frequently used as template for real-time PCR standards and with 3 different size magnitudes (ssDNA – 104 nucleotides, plasmid DNA – ~3,1 kb and genomic DNA – ~4,6 mb) and the seal type (8-cap strip / adhesive film and with or without 7 µl of mineral oil) on the relative increase of the DNA concentration as an indication for co-evaporation of DNA with water after 1 and 5 real-time PCR runs is shown in Fig. 3.1.

After 1 run a relative increase in the DNA concentration in all wells was noticed, regardless of the DNA type. However, this increase was significantly higher in wells with genomic DNA (15.11 %) in comparison to wells with ssDNA (10.93 %) and plasmid DNA (10.38 %). These

observations suggest that evaporation of water occurred in all wells. The differences in the relative increase of the DNA concentration suggest the co-evaporation of DNA, to a lesser degree in wells with genomic DNA and to a greater extent in wells with ssDNA and plasmid DNA. After 5 runs similar observations were noticed, but as expected the relative increase of DNA concentrations in all wells was higher than after 1 run. Although the relative increase in the DNA concentration was again highest in wells with genomic DNA (27.64 %), there was also a significant difference between the ssDNA fragments (20.50 %) and the plasmid DNA (24.47 %). An apparent correlation between the size of the DNA and the increase of the DNA concentration was noticeable (Fig 3.1).

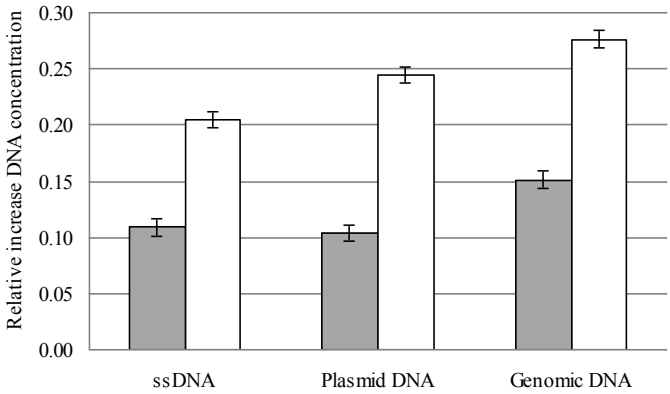

Fig. 3.1: Effect of the DNA type (horizontal axis) on the relative increase ((final concentration – initial concentration) x100 / initial concentration; vertical axis) of the DNA concentration after (▣) 1 run and after (□) 5 runs. Each DNA type in the horizontal axis represents all possible combinations of (1) a specific DNA type (ssDNA, plasmid DNA, genomic DNA) with (2) all combinations of two seal types (8-cap strip or adhesive film) and whether or not 7 µl of mineral oil was used. Vertical error bars denote the 95% confidence intervals.

Regardless of the seal type and whether or not 7 µl of mineral oil was used as cover on the DNA solution, a relative increase in the DNA concentration in all wells was noticed after 1 run (Fig. 3.2). A significantly higher relative increase in DNA concentration in all wells covered by the mineral oil was noticed (15.40 % and 13.90 % vs. 9.54 % and 9.72 %). This may suggest that mineral oil, although permitting the evaporation of water may have prevented to some extent the co-evaporation of DNA as such resulting in the increased DNA concentration in the well. After 5 runs (Fig 3.2) a significantly higher relative increase in DNA concentration was noticed in wells sealed by the 8-cap strip (29.30 % and 33.48 %) in comparison to the wells sealed by the adhesive film (15.73 % and 18.30 %). Although the increase in DNA

concentration was consistently higher in wells where mineral oil was used, this difference was not significant.

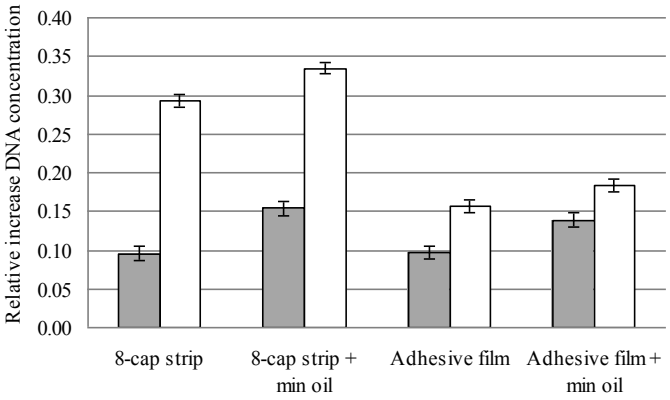

Fig. 3.2: Effect of the seal type (horizontal axis) on the relative change ((final concentration – initial concentration) x100 / initial concentration; vertical axis) of the DNA concentration after ( ) 1 run and after ( ) 5 runs. Each seal type in the horizontal axis represents all possible combinations of (1) the combinations of a seal type (8-cap strip or adhesive film) and whether or not 7 µl of mineral oil was used with (2) the three DNA types (ssDNA, plasmid DNA and genomic DNA). Vertical error bars denote the 95% confidence intervals.

Given the results of the "evaporation-experiment", the effect of different amounts (7 and 30 µl) of mineral oil as vapor barrier on the efficiency of real-time PCR reactions was tested in comparison to when no mineral oil was used. A real-time PCR amplification was performed in which duplicates of a 10-fold serial diluted series of the ssGII fragment were taken as standard positive control. This real-time PCR was run twice independently and parameter values of the standard curves are shown in Table 3.2. Seven µl of mineral oil did not cause a considerable reduction of the sensitivity, reproducibility and efficiency of the real-time PCR assay, while this was not the case when 30 µl was used.

Table 3.2: **Parameter values of the standard curves when different amounts of mineral oil were used as cover layer.**

Volume mineral oil	**Detection limit (Ct)**	**Slope (PCR-efficiency)**	**R^2-value**
0 µl	10 copies (40.38)	-3.36 (98.5%)	0.99
7 µl	10 copies (39.51)	-3.64 (88.3%)	1.00
30 µl	10 copies (42.5)	-2.01 (214.4%)	0.432

To verify the effect of the seal type (8-cap strip and adhesive film) on the occurrence of positive NTCs, two independent real-time PCR runs as described above were performed, with duplicates of a 10-fold diluted standard series of ssGII as standard positive control and with either the 8-cap strip or the adhesive film as seal type. The use of the 8-cap strip seemed to reduce number of positive NTCs (1/16) compared to when the adhesive film was used (all 4 NTCs positive, with Ct values corresponding to an original concentration of 10 copies). However, the 8-cap strip seemed to reduce the reproducibility of the assay, resulting in a low R^2-value (0.834). The use of the adhesive film resulted in a higher R^2-value (0.997).
The GII NoV real-time PCR was then carried out by using pGII as 10-fold diluted standard positive control (10^5-10 copies), an adhesive film as seal type and 7 µl of mineral oil as cover layer on top of the PCR reaction mixtures (figure 3). An efficient (PCR-efficiency of 101.78%) , reproducible (R^2-value = 1) and sensitive (detection limit of 10 copies) real-time PCR assay was observed while no amplification occurred in any of the 18 NTCs. A standard curve with a detection limit of 10 copies (with intercept 42.82), a R^2-value of 1 and a slope of -3.28 (corresponding to a PCR-efficiency of 101.78%) indicated that the chosen setup does not have a negative influence on the performance of the real-time PCR assay.

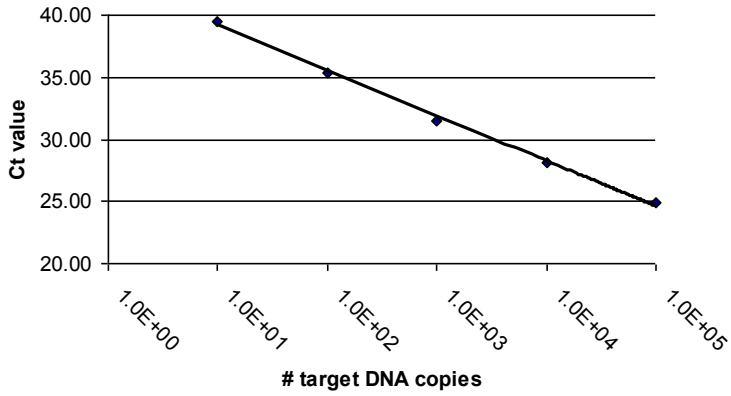

Fig. 3.3: Standard curve for the real-time PCR assay using following setup: (1) 10-fold serial diluted pGII plasmid was used as standard ranging from 10^5 to 10^1 copies, (2) the real-time PCR reaction plate was sealed using an adhesive film and (3) seven µl of mineral oil was used as cover layer on top of the real-time PCR reaction mix.

In summary, the above results show that it is recommended to use larger DNA molecules, such as plasmids instead of ssDNA fragments to generate standard curves as positive control in real-time PCR. Moreover, 7 µl of mineral oil on top of the real-time PCR reaction mix attributed to prevent positive NTCs. Since the reduced reproducibility of the real-time

PCR assay did not compensate for the reduced number of positive NTCs when the 8-cap strip was applied as seal type, the adhesive film was chosen as the preferred seal type.

3.5. Discussion

DNA contamination is a reported drawback of conventional and real-time PCR (Josefsson et al., 1999). It has been stated that the risk of DNA contamination has decreased by real-time PCR, due to the closed system which avoids the necessity for the post-PCR handling of amplified material (Klein, 2002; Mackay et al., 2002). Additional systems such as uracil N-glycosylase (UNG) are known methods to prevent carryover contamination of (real-time) PCR amplified material (Pang et al., 1992; Pruvost et al., 2005). However, our results show that these systems do not solve all contamination issues.

A frequent occurrence of positive NTCs was noticed when a ssDNA fragment (ssGII) was used as real-time PCR standard. In contrast, no amplification occurred in any of the NTCs when a plasmid (pGII) was used as standard positive control. Only minor differences in sensitivity were noticed when ssDNA fragments or plasmid DNA were used as real-time PCR standard, confirming previous studies (Moriya et al., 2006). The hypothesis of the positive NTCs being caused by co-evaporation of DNA with water resulting in well-to-well migration of DNA during the real-time PCR was raised and investigated.

Data obtained from the “evaporation-experiment” indicated that evaporation of water and co-evaporation of DNA occurred during a real-time PCR run regardless of the DNA type, seal type (adhesive film and 8-cap strip) or the use of 7 µl of mineral oil as cover layer. An apparent negative correlation between the size of the DNA and the extent of the co-evaporation of the DNA was also noticeable, suggesting the use for larger DNA molecules, such as plasmids instead of ssDNA fragments as standard in real-time PCR.

The higher relative increase in DNA concentration observed when mineral oil was used as cover layer suggests that the mineral oil prevented to some extent the co-evaporation of DNA. A similar conclusion – although to a lesser degree – can be drawn when 8-cap strips were used as seal type.

Given the results of the “evaporation”- experiment, the effect of 2 seal types (adhesive film and 8-cap strip) on the occurrence of positive NTCs was examined. Furthermore, the effect of different amounts of mineral oil on the performance of the real-time PCR was tested. The use of mineral oil and paraffin wax has been suggested before when trying to prevent false-positive PCR results (Rijpens and Herman, 2002; Sparkman, 1992).

The great sensitivity of optimized real-time PCR formats is responsible for the increasing number of detection assays using this technique (Valasek and Repa, 2005). Nevertheless, this high sensitivity should also be considered as a potential risk in the use of high-sensitive techniques because a minor contamination results in positive NTCs (Mobius et al., 2008).

3.6. Conclusions

Results obtained from this chapter showed that it remains necessary to take appropriate measures to gather reliable results from real-time PCR assays, as different factors can influence the outcome of real-time PCR experiments. A constant awareness should also be focused on the people executing PCR and in the interpretation of results. These measures are important towards detection of NoV in foods, as NoV levels close to the detection limit are expected.

3.7. Acknowledgements

The authors would like to thank Ann Vanhee and Jessy Claeys for the excellent technical assistance and Dr. Winy Messens for the statistical analysis of the “evaporation experiment”-data.

Chapter 4

EVALUATION OF A NOROVIRUS DETECTION METHODOLOGY FOR READY-TO-EAT FOODS.

Redrafted after

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4. CHAPTER 4: EVALUATION OF A NOROVIRUS DETECTION METHODOLOGY FOR READY-TO-EAT FOODS.

4.1. Abstract

Despite recent norovirus (NoV) foodborne outbreaks related to consumption of ready-to-eat (RTE) foods, a standardized assay to detect NoV in these foods is not available yet. Therefore, the robustness of a methodology for NoV detection in RTE foods was evaluated. The NoV detection methodology consisted of direct RNA extraction with an eventual concentration step, followed by RNA purification and a multiplex real-time reverse transcriptase (RT-) PCR assay for the detection of GI and GII NoV and the murine norovirus-1 (MNV-1), the latter used as process control. The direct RNA extraction method made use of the guanidine-isothiocyanate containing reagent (Tri-reagent®, Ambion) to extract viral RNA from the food sample (basic protocol called TriShort), followed by an eventual concentration step using organic solvents (extended protocol called TriConc).

To evaluate the robustness of the NoV detection method, the influence of (1) the NoV inoculum level and (2) different food types on the recovery of NoV from RTE foods was investigated. Simultaneously, the effect of two RNA purification methods (manual RNeasy minikit (Qiagen) and automated NucliSens EasyMAG (BioMérieux)) on the recovery of NoV from these foods was examined. Finally, MNV-1 was evaluated as process control.

First of all, high level GI and GII NoV inocula (~10^6 NoV genomic copies / 10 g) could be recovered from penne salad samples (10 g) in at least 4 out of 6 PCRs, while low level GI and GII NoV inocula (~10^4 NoV genomic copies / 10 g) could be recovered from this food product in maximally 3 out 6 PCRs, showing a significant influence of the NoV inoculum level on its recovery.

Secondly, low level GI and GII NoV inocula (10^4 NoV genomic copies / 10 g) were spiked onto 22 ready-to-eat food samples (10 g) classified in three categories (soups, deli sandwiches and composite meals). The GI and GII NoV inocula could be recovered from 20 of the 22 samples.

The TriConc protocol provided better recoveries of GI and GII NoV for soups while the TriShort protocol yielded better results for the recovery of GII NoV from composite meals. NoV recovery from deli sandwiches was problematic using either protocol.

Thirdly, the simultaneous comparison of two RNA purification protocols demonstrated that automated RNA purification performed equally or better compared to manual RNA extraction.

Finally, MNV-1 was successfully evaluated as process control when detecting NoV in RTE foods using this detection methodology.

In conclusion, the evaluated NoV detection method was capable of detecting NoV in RTE foods, although recoveries were influenced by the inoculum level and by the food type.

4.2. Introduction

Viral pathogens such as NoV are frequently transmitted through food and water and have been reported to cause food borne gastroenteritis outbreaks (Goyal, 2006). Fat and protein based foods such as deli sandwiches (De Wit et al., 2007; Payne et al., 2006; Sala et al., 2005), prepared meals (Ohwaki et al., 2009; Parasidis et al., 2007), buffet foods (Boxman et al., 2007) and restaurant lunches (Bohm et al., 2008; Hirakata et al., 2005) have been considered as the causative food vehicles in described food borne NoV outbreaks. Contamination of these foods is in most cases caused by an infected food handler (Baert et al., 2009b).

Currently, no reliable method is available for cultivation of NoV (Duizer et al., 2004) and detection of this pathogen in foods relies exclusively on molecular methods. However, molecular detection of food borne pathogens on food samples is – compared to clinical samples – hardened due to low levels of virus particles and the presence of inhibiting substances (McKillip and Drake, 2004; Rijpens and Herman, 2002).

Therefore, a number of virus detection methods have been described combining (1) a protocol for extraction and concentration of (genomic material of) NoV while reducing the presence of inhibitory substances, (2) an RNA purification method and (3) a molecular detection method. Examples of described viral extraction-concentration for RTE foods protocols are based on the elution – concentration principle (Kobayashi et al., 2004; Leggitt and Jaykus, 2000; Papafragkou et al., 2008; Rutjes et al., 2006a) or on the direct extraction of RNA from the food sample (Boxman et al., 2007; Gouvea et al., 1994; Schwab et al., 2000). Real-time RT-PCR is currently considered as the molecular method of choice for the detection of NoV in clinical, food and environmental samples (Jothikumar et al., 2005b; Park et al., 2008; Wolf et al., 2007). Currently, most labs are restricted to their in-house developed virus detection methods for these food types since a standardized virus detection method is not available and limited attention has been spent on the evaluation and validation of developed methods.

Therefore, the current chapter of this PhD dissertation describes the robustness evaluation of a proposed method for the detection of NoV in RTE foods consisting of (1) a direct RNA extraction protocol described by Baert et al (2008a) and (2) a multiplex real-time RT-PCR described in chapter 2 of this PhD dissertation. The influence of the NoV inoculum level on its recovery was determined by inoculating 2 levels of GI and GII NoV on penne carbonara salad samples, while the influence of the food type was analyzed by inoculating low levels of GI and GII NoV on a broad range of RTE foods including soups, deli sandwiches and

composite meals. Simultaneously, the influence of 2 RNA purification methods (the manual RNeasy minikit (Qiagen) and the automated NucliSens EasyMAG (BioMérieux)) on the recovery of NoV detection was investigated. Meanwhile, MNV-1 was tested as process control for detection of NoV from RTE foods.

4.3. Materials and Methods

4.3.1. Food samples used for evaluation of the virus extraction method

A penne carbonara salad (450 g) was purchased at a local food store and 22 food products (4 soups, 4 deli sandwiches and 14 composite meals; Table 1) were provided by a canteen for local students and personnel at Ghent University. The composite meals were samples with combinations of several ingredients containing (1) meat (or meat replacement), (2) vegetables and (3) potato puree or rice in a 3:3:4 ratio except in two cases (Table 1).
All food samples were divided in 10 g aliquots and kept at -20°C until used.

Table 4.1 Overview of the food samples used for the evaluation of the influence of the food type on virus recovery. Components 1, 2 and 3, respectively were combined in a 3:3:4 ratio. In two cases (marked with *), only two components were combined in a 1:1 ratio. All food samples (10 g) were inoculated with a combined GI NoV, GII NoV and MNV-1 inoculum.

	Component 1	Component 2	Component 3
Composite meals	Fish pie	Red cabbage	Potato puree
	Heekfilet delight	Beans	Potato puree
	Fish (HM)	Cauliflower + cheese sauce	Boiled potatoes
	Chicken casserole	Brussels Sprouts	Boiled potatoes
	Turkey	Cream savoye cabbage	Potato puree
	Ham rolls	Cream savoye cabbage	Potato puree
	Meatballs with tomato sauce	Peas	Potato puree
	Pig goulash	Vichy carrots	Rice
	Champion à la Grecque*	Potato salad*	/
	Tomato-Mozarella*	Potato puree*	/
	Lasagna	/	/
	Spirelli with vegetables	/	/
	Rice with vegetables	/	/
	Boulgoursalad with shellfish	/	/
Soups	Broccoli soup		
	Creamy mushroom soup		
	Tomato soup		
	Creamy chicken soup		
Deli sandwiches	Cereal deli sandwich with chicken-curry salad		
	Cereal deli sandwich with chicken-andalouse salad		
	Deli sandwich with crab salad		
	Deli sandwich with Americain prepare		

4.3.2. Artificial contamination of food samples

Food samples were artificially contaminated with GI and/or GII NoV and/or murine norovirus 1 (MNV-1). For the GI and GII NoV inoculations, 2 stool samples containing GI.2 and GII.4 NoV were provided by the Rega Institute for Medical Research (Leuven, Belgium). Additionally, the food samples were artificially contaminated with 1000-fold diluted MNV-1 virus lysate (Baert et al., 2008b). Tenfold serial dilutions of both faecal samples and of the MNV-1 virus lysate were prepared in PBS (145 mM NaCl, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.4; Cellgro, Mediatech Inc., USA) and stored at -80°C until use. GI and/or GII NoV inoculated food samples were incubated overnight at 4°C to allow attachment of GI/GII NoV to the foods, while the incubation time for MNV-1 inoculation was 30 min. The latter incubation time was chosen to reduce the duration of the protocol when testing food products for NoV. Concentrations of GI/GII NoV and MNV-1 inocula were determined by Real-time RT-PCR.

To investigate the influence of the NoV inoculum level on its recovery, 2 different levels of GI and GII NoV were inoculated onto a total of 8 penne salad samples (10 g). The high level GI and GII NoV inocula contained respectively 1.40×10^6 and 5.61×10^5 NoV genomic copies, while the low level GI and GII NoV inocula contained 4.02×10^3 and 4.91×10^4 NoV genomic copies.

In detail, high and low GI and GII NoV inocula were each inoculated separately on a penne salad sample and in every possible combination on another 4 samples. A high level MNV-1 inoculum (1.41×10^6 genomic copies) functioning as process control was added to every inoculated penne salad. After direct RNA extraction by the TriShort and TriConc protocols, RNA was purified either manually or automated and every PCR was duplicated. Consequently, every inoculum (GI or GII NoV, high or low) was spiked onto 3 of the 8 samples (and the recovery was thus tested by 12 PCR reactions: 6 PCR reactions for the automated RNA purification and 6 PCR reactions for the manual RNA purification). Recovery of the MNV-1 inoculum was tested by 32 PCR reactions (16 PCR reactions for the automated RNA purification and 16 PCR reactions for the manual RNA purification) in all 8 samples.

To analyze the influence of different food types on the recovery of GI/GII NoV and MNV-1, 10 g samples of three categories of RTE foods (4 deli sandwiches, 4 soups and 14 composite meals) were inoculated with a combined low level GI NoV, low level GII NoV and high level MNV-1 inoculum (the latter serving as process control). The low level GI and GII NoV inocula contained 1.28×10^4 and 5.81×10^4 NoV genomic copies while the high level MNV-1 inoculum contained 1.35×10^7 genomic copies. Every PCR was duplicated. Consequently, the GI NoV, GII NoV and MNV-1 inocula (extracted by either the TriConc or TriShort protocol and RNA purified either manually or automated) were each detected by 8 PCR reactions for

the deli sandwiches (4 samples), 8 PCR reactions for the soups (4 samples) or by 28 PCR reactions for the composite meals (14 samples).

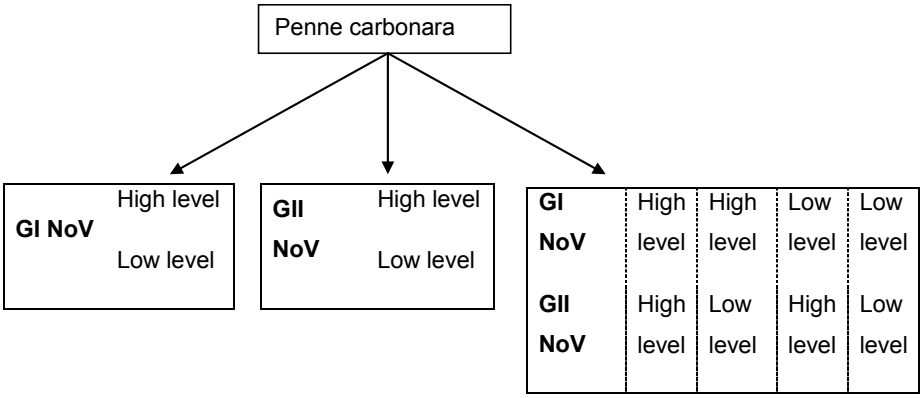

Fig. 4.1 Overview of the inoculation scheme used for investigation of the influence of the NoV inoculum level on its recovery.

4.3.3. Virus extraction

The virus extraction method was based on a previously reported method (Baert et al., 2008a). Briefly, 10 g of food product was homogenized in a 50 ml centrifuge tube with 8 ml of Tri Reagent® (Ambion Inc, Texas, USA) allowing a contact time of 20 min while shaking at room temperature at 200 rpm (IKA®-werke, Staafen, Germany). After centrifugation (12,000 × g, 10 min, 4 °C), the nucleic acid extract (supernatant) was transferred to a 15 ml centrifuge tube and subsequently processed. The volume of this nucleic acid extract varied between 3 and 12 ml, depending on the food type tested. According to the processing procedure of this nucleic acid extract, protocols were named “TriShort” and “TriConc”.

“TriShort”: RNA purification was performed on 100 µl of the nucleic acid extract.

“TriConc”: the remaining nucleic acid extract was transferred to 2 ml microcentrifuge tubes and subjected to a second concentration/purification procedure described by the product descriptions of Tri Reagent®. Shortly, 800 µl of chloroform (VWR International, Leuven, Belgium) was added per ml nucleic acid extract and mixed for 15 s followed by 2–3 min settling before centrifugation (10,000 × g, 15 min, 4 °C). Isopropanol (Sigma-Aldrich; Steinheim; Germany) was added to the aqueous phase and samples were placed at 4 °C for 10 min. RNA was precipitated by centrifugation (9503 × g, 10 min, room temperature). The precipitated RNA was washed with 75% of room temperature ethanol (Merck, Leuven, Belgium) and subsequently with acetone (Merck). The washing solution was removed after each washing step by centrifugation (9503 × g, 5 min, room temperature). Finally, the pellet

was dried and then dissolved in 100 µl of nuclease free water (Qiagen, Hilden, Germany) for subsequent RNA purification.

4.3.4. RNA purification

Purification of the RNA was performed by the use of the manual RNeasy Mini kit (Qiagen) using the RNA Cleanup protocol (elution volume = 30 µl) or by the automated NucliSens EasyMAG system (BioMérieux, Marcy l'Etoile, France) using the protocol according to the manufacturer's instructions (elution volume = 25 µl).

4.3.5. Reverse transcription

A pre-reaction mix consisting of 3 µl of extracted and purified RNA, 1 µl of random hexamers (50 µM; Applied Biosystems, Foster City, CA) and nuclease-free water in a final volume of 11.5 µl was heated to 95°C during 2 min followed by 2 min cooling on ice. This first pre-reaction mix was then mixed with a second pre-reaction mix of 8.5 µl to obtain a final 20 µl reverse transcription (RT-) mastermix containing 2.5 µM random hexamers (Applied Biosystems), 25 U of Multiscribe reverse transcriptase (Applied Biosystems), 20 U of RNase inhibitor (Applied Biosystems), 5 mM $MgCl_2$ (Applied Biosystems), 1×PCR buffer II (10 mM Tris HCl pH 8.3 and 50 mM KCl; Applied Biosystems), 1 mM dNTPs (GE Healthcare; Diegem, Belgium) and RNA. Reverse transcription was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) and the temperature profile included 10 min at 22°C, 15 min at 42°C, 5 min at 99°C and 5 min at 5°C. All cDNA was stored at -20°C.

4.3.6. Real-time PCR .

Multiplex real-time PCR for simultaneous detection of GI and GII NoV and MNV-1 was carried out as described in chapter 2. Quantification, collection of amplification data and data analysis were performed using the LC480II real-time PCR instrument (Roche Diagnostics, Mannheim, Germany).

All real-time PCR reactions were duplicated.

4.3.7. Data analysis

The recovery of GI and GII NoV and MNV-1 from the food samples was both qualitatively and quantitatively analyzed. Firstly, qualitative analysis of the recovery of the GI and GII NoV and MNV-1 inocula from food samples was defined as the "recovery success rate". This recovery success rate was calculated as "the number of PCR reactions showing successful recovery of GI/GII NoV or MNV-1" per "number of PCR reactions performed". Secondly, quantitative analysis of the recovery of GI and GII NoV or MNV-1 from individual food samples was defined as "recovery efficiency". This recovery efficiency (%) was calculated

per individual sample as “the mean recovered number of GI and GII NoV or MNV-1 genomic copies” per “mean inoculated number of GI/GII NoV or MNV-1 genomic copies“.
Statistical analysis of the recovery success rates was performed using the non-parametric Mantel and Haezel test (MH-test) or the non-parametric Kruskal-Wallis test (KW-test). All statistical analyses were performed using the PASW Statistics 18 software (SPSS Inc., Chicago, IL). Significance levels were set at 0.05.

4.4. Results

4.4.1. Influence of the NoV inoculum level and RNA purification method on virus recovery.

Qualitative data showed that the presence of GI NoV did not significantly influence the recovery success rates of GII NoV and vice versa, regardless whether the TriShort or TriConc protocol was applied and regardless whether the manual or automated RNA purification was used (KW-test; p = 0.212 and p = 0.768, respectively). Therefore, the qualitative results shown in Table 2 were combined per NoV inoculum (and thus per six PCR reactions).

Table 4.2 Overview of the qualitative results obtained when evaluating the influence of the virus inoculum level on the recovery of GI NoV, GII NoV and MNV-1 from penne salad.

Inoculum	Inoculum level[a]	RNA purification[c]	Recovery success rate[d]	
			TriShort	TriConc
GI NoV	1.40×10^6	manual	5/6	6/6
		automated	6/6	6/6
	4.02×10^3	manual	0/6	3/6
		automated	1/6	0/6
GII NoV	5.61×10^5	manual	5/6	6/6
		automated	4/6	6/6
	4.91×10^4	manual	0/6	1/6
		automated	0/6	1/6
MNV-1[e]	1.41×10^6	manual	15/16	16/16
		automated	16/16	16/16
Negative control	/	manual	nd[b]	nd
		automated	nd	nd

[a] expressed as NoV genomic copies per 10 g penne salad. [b] not detected. [c] manual: RNeasy minikit (Qiagen), automated: NucliSens EasyMAG (BioMérieux). [d] # positive real-time PCR reactions / # performed real-time PCR reactions. [e] MNV-1 added to the penne salad samples was prepared as a virus lysate as described by (Baert et al., 2008b).

The inoculum level of GI and GII NoV had a significant influence on the respective recovery success rates, regardless whether the TriShort or TriConc protocol was applied and regardless whether the manual or automated RNA purification was used (KW-test; $p < 0.001$ for both GI and GII NoV).

Qualitative data also showed that the recovery success rates of the TriConc protocol were significantly higher compared to the success rates of the TriShort protocol (MH-test; $p=0.009$), while no significant differences could be shown between the recovery success rates obtained when the RNA was purified manually or automatically (MH-test; $p = 0.681$ and 0.082 for the TriShort and TriConc protocols, respectively).

Table 4.3 Overview of the quantitative results obtained when evaluating the influence of the virus inoculum level on the recovery of GI NoV, GII NoV and MNV-1 from penne salads.

	TriShort				
	GI NoV[c]		GII NoV[c]		MNV-1[c]
Recovery efficiency[a]	1.40×10^6	4.02×10^3	5.61×10^5	4.91×10^4	1.41×10^6
> 10 %	1/12	1/12	3/12		14/32
1 % - 10 %	8/12		5/12		17/32
0.1 % - 1 %	2/12				
No recovery	1/12	11/12	4/12	12/12	1/32
Mean recovery ± stdev[b]	3.8 % ± 3.9 %	49.0 %	11.5 ± 7.3 %		11.8 % ± 10.1 %
	TriConc				
	GI NoV[c]		GII NoV[c]		MNV-1[c,d]
Recovery efficiency[a]	1.40×10^6 gen cop /10g	4.02×10^3 gen cop /10g	5.61×10^5 gen cop /10g	4.91×10^4 gen cop /10g	1.41×10^6 gen cop /10g
> 10 %		2/12	2/12		6/32
1 % - 10 %	2/12	1/12	3/12	2/12	15/32
0.1 % - 1 %	10/12		6/12		11/32
No recovery		9/12	1/12	10/12	
Mean recovery ± stdev[b]	1.0 % ± 1.5 %	12.0 % ± 8.8 %	3.3 % ± 5.6 %	8.4 % ± 1.3 %	4.2 % ± 4.8 %

[a] ("mean recovered number of GI/GII NoV or MNV-1 genomic copies" / "mean inoculated number of GI/GII NoV or MNV-1 genomic copies") × 100 %.

[b] stdev: standard deviation

[c] inoculum level expressed as genomic copies / 10 g food sample.

[d] MNV-1 added to the penne salad samples was prepared as a virus lysate as described by (Baert et al., 2008b).

Quantitative data (Table 3) showed that the TriShort protocol resulted in a recovery of the high level GI and GII NoV inocula with efficiencies of >1 % in most PCR reactions (70.8 %). On the other hand, the TriConc protocol resulted in recovery efficiencies between 0.1 % and 10 % for these inocula in most PCR reactions (87.5 %). Of the low level GI and GII Nov inocula, only a single PCR reaction yielded a positive result when the TriShort protocol was used, while this was the case for 5 PCR reactions if the TriConc protocol was applied. Finally, the MNV-1 inoculum was recovered in all but one case (Table 2 and 3).

4.4.2. Influence of the food type on virus recovery.

The low level GI and GII NoV inocula could both be detected in 20 of the 22 samples using either the TriShort or TriConc protocol (Table 4). In detail, The GI NoV inoculum could not be recovered from the rice with vegetables dish, and the recovery of the GII NoV inoculum was not possible from the dish with champignon à la Grecque and potato salad. The recovery success rates for the GI and GII NoV inocula were (both for TriShort and TriConc protocols) significantly higher for the composite meals compared to soups or deli sandwiches, regardless of the used RNA purification method (Table 4; MH-test: all p-values < 0.024).

Table 4.4 Overview of the qualitative results obtained when evaluating the influence of the food type (4 deli sandwiches, 4 soups and 14 composite meals) on virus recovery.

Inoculum	Inoculum level[a]	RNA isolation[b]	Recovery success rate					
			Deli sandwiches (n=4)		Soups (n=4)		Composite meals (n=14)	
			Tri short	Tri conc	Tri short	Tri conc	Tri short	Tri conc
GI NoV	1.28×10^4	Manual	1/8 [c]	2/8	1/8	2/8	7/28	5/28
		Automated	2/8	0/8	4/8	7/8	11/28	18/28
GII NoV	5.81×10^4	Manual	1/8	3/8	1/8	3/8	8/28	4/28
		Automated	1/8	1/8	5/8	7/8	12/28	8/28
MNV-1	1.35×10^7	Manual	8/8	8/8	8/8	8/8	28/28	28/28
		Automated	8/8	7/8	8/8	8/8	28/28	28/28

[a] expressed as NoV genomic copies per 10g food. Every sample was inoculated with the combined GI NoV, GII NoV and MNV-1 inoculum.

[b] manual: RNeasy minikit (Qiagen), automated: NucliSens EasyMAG (BioMérieux)

[c] # positive real-time PCR reactions / # performed real-time PCR reactions

For soups, both the automated RNA purification and TriConc protocol provided significantly better results compared to the manual RNA purification and TriShort protocol, respectively (MH-test; $p<0.001$ and $p=0.025$, respectively). Automated RNA purification generated in the composite meals provided significantly better results compared to the manual RNA purification, while the virus extraction method (TriShort or TriConc) did not affect the results significantly (MH-test; $p<0.001$ and $p=0.562$, respectively). Recovery of the GI and GII NoV inocula in deli sandwiches was difficult, regardless of the used virus extraction protocol or RNA purification method. The high level MNV-1 inoculum could be detected in all PCR reactions, except in one case (Table 4).

4.5. Discussion

In the current study, a method for detection of NoV in RTE foods was evaluated, consisting of (1) a direct RNA extraction method (Baert et al., 2008a) and (2) a multiplex real-time RT-PCR as described in chapter 2 of this PhD dissertation.

The direct RNA extraction step contained two subsequent protocols wherein an initial RNA extraction (protocol name: TriShort) is followed by an eventual concentration/purification of the extracted RNA (protocol name: TriConc).

First of all, the effect of the NoV inoculum level on the recovery of NoV from foods was investigated by spiking high and low concentrated GI and GII NoV inocula (respectively ~10^6 and ~10^4 and genomic copies) onto 10 g penne salad samples, a selected RTE food type. Since a cultivation system is not available for GI and GII NoV, it was not possible to relate these genomic copy numbers to the number of infectious virus particles. However, a ratio of 30 to 333 genomic copies per infectious virus particle has been observed for the murine norovirus 1 (MNV- 1), coxsackievirus B4, poliovirus 1 and 2, enterovirus and hepatitis A virus HM 175 (Baert et al., 2008c; de Roda Husman et al., 2009; Donia et al., 2010; Mullendore et al., 2001). Therefore, a GI and GII NoV load of 10^4 genomic copies per 10 g of food product could hypothetically correspond to a load of 30 to 300 infectious GI/GII NoV particles per 10 g, although further research is needed as several factors such as temperature and pH can severely influence this ratio.

In general, the NoV detection method was able to recover the high level NoV inocula from penne salad with varying recovery efficiencies (0.1 % to > 10 %), while the low level NoV inocula proved to be difficult to recover from this food type. A similar protocol combining the use of TRIzol® Reagent with an additional RNA purification step has been able to recover 10^4 RT-PCR units of NoV per 40 g of ham and turkey meat with a ~1 % efficiency, while no recovery was possible in roast beef meat (Schwab et al., 2000). In another study, 2.5×10^5 $TCID_{50}$ of canine Calicivirus (CaCV) were inoculated on lettuce and whipped cream and could be recovered with recoveries ranging between 1 % and 10 %, using a NaCl-PEG

precipitation step in addition to a TRIzol®-based direct RNA extraction protocol (Rutjes et al., 2006a). The latter study also demonstrated a 1 % recovery efficiency of the CaCV inoculum in macaroni, a food product similar to the penne salad. Recovery success rates of the high level NoV inocula were significantly higher compared to the low level NoV inocula. In agreement with the presented results, Cheong et al (2009a) demonstrated that the recovery of NoV in lettuce was more successful at high concentrations, both in large and small volumes of tested food samples.

Secondly, the influence of the food type on the recovery of NoV was investigated by inoculating a broad range of RTE foods. Recovery of the inocula from deli sandwiches was difficult, which can most likely be explained by the absorbing properties of the food matrix (lowering the recovered volume of nucleic acid extract). However, a 1 % recovery of a CaCV inoculum from white bread has been observed before using a similar protocol, in combination with a NaCl-PEG precipitation (Rutjes et al., 2006). Nevertheless, an investigation of NoV outbreaks related to the consumption of deli sandwiches showed that in most cases, NoV could not be detected in these RTE food products (Daniels et al., 2000; de Coster et al., 2001; De Wit et al., 2007; Dominguez et al., 2008; Payne et al., 2006; Sala et al., 2005). Further research might therefore in particular be recommended to improve the recovery of low level NoV inocula from this food type.

Thirdly, comparison of two RNA purification methods as part of the NoV detection method showed that both systems performed equally when recovering high and low level NoV inocula from penne salad samples. For soups and composite meals, the automated NucliSens EasyMAG system (BioMérieux) generated significantly better results when recovering low level GI and GII NoV inocula compared to the manual RNeasy Mini kit (Qiagen). While some authors have reported reduced recovery of foodborne viruses from produce using automated RNA purification (Butot et al., 2007; Morales-Rayas et al., 2010), advantages of automated and manual RNA purification methods when detecting viral pathogens have been described before (Witlox et al., 2008).

Finally, the high level MNV-1 inoculum was successfully evaluated as process control when detecting NoV in RTE foods as it could be recovered in all food types tested. The use of MNV-1 as process control has been evaluated successfully in soft red fruits (described in chapter 5 of this PhD dissertation).

In conclusion, the TriShort and TriConc protocols are, in combination with a sensitive Real-time RT-PCR assay, capable of reliably detecting high concentrations (~10^6 genomic copies / 10 g food sample) of NoV in RTE foods, while detection of lower NoV levels (~10^4 genomic copies / 10 g food sample) was more difficult. However, as outbreaks can be caused by only 10-100 NoV particles, current detection methods may still lack the necessary sensitivity to detect these very low concentrated viral agents in the suspected foods. This may explain

why reported viral foodborne outbreaks are based on epidemiological data rather than on actual detection of the viral particles in the food (Nordgren et al., 2010b; Ohwaki et al., 2009). Except for soups, both protocols did not generate significantly different results. Therefore, the more laborious TriConc protocol could function as an additional virus extraction when samples tested negative using the TriShort protocol. However, a food sample testing negative for NoV does not automatically mean total absence of NoV, due to the observed limitations of the presented method (in particular with deli sandwiches). On the other hand, it is recommended to confirm samples that tested positive by (real-time) RT-PCR for NoV. This confirmation can be obtained by sequencing specific NoV genomic regions, although problems such as aspecific amplification products as well as inhibition can be encountered frequently during these genotyping efforts. Therefore, detection of NoV in foods, and in particular in RTE foods, is an area that still requires further research.

4.6. Conclusions

In conclusion, chapter 4 of this PhD dissertation described the evaluation of a direct RNA extraction protocol as part of a method for detection of NoV in RTE foods such as pasta salads, composite meals, deli sandwiches and soups. Results showed that the TriShort and TriConc protocol variants are, in combination with the multiplex real-time RT-PCR assay described in chapter 2 of this PhD dissertation, capable of reliably detecting high concentrations (10^5 to 10^6 genomic copies / 10 g food sample) of NoV in these foods, while detection of low concentrated NoV levels was influenced by the food type. Nevertheless, aided by a few modifications towards difficult matrices such as deli sandwiches, this method could be used for analysis of foods implicated for NoV presence.

4.7. Acknowledgements

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Chapter 5

EVALUATION OF A NOROVIRUS DETECTION METHODOLOGY FOR SOFT RED FRUITS.

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5. CHAPTER 5: EVALUATION OF A NOROVIRUS DETECTION METHODOLOGY FOR SOFT RED FRUITS.

5.1. Abstract

In the present chapter, an elution-concentration methodology for detection of genogroup I (GI) and II (GII) noroviruses (NoV) in soft red fruits was evaluated. The murine norovirus 1 (MNV-1), a recently described cultivable genogroup V NoV was integrated in the detection methodology as full process control (MNV-1 PC), reverse transcription control (MNV-1 RTC) and real-time PCR internal amplification control (MNV-1 IAC). Both the sensitivity and robustness of the proposed methodology were analyzed.

Firstly, the sensitivity of the method was examined by analysis of the recovery of MNV-1, GI and/or GII NoV inoculated on frozen raspberry crumb samples. Results showed that the recovery of MNV-1 was not significantly influenced by the inoculum incubation time (30 min or overnight incubation) or the inoculum level (10^6 or 10^8 MNV-1 genomic copies/10 g of frozen raspberry crumb sample). In contrast, a significant influence of the GI and GII NoV inoculum level (10^4 or 10^6 genomic MNV-1 copies/10 g of frozen raspberry crumb sample) was noticed on the recovery of respectively GI and GII NoV from frozen raspberry crumb samples.

Secondly, the robustness of the methodology was evaluated by subjecting three types of artificially MNV-1, GI and/or GII NoV contaminated soft red fruit products (deepfrozen forest fruit mix, fresh raspberries and fresh strawberry puree) to the method. Results showed a significant influence of the soft red fruit product type on the recovery efficiency of GI NoV and MNV-1, while no significant differences could be shown for GII NoV. In general, the recovery of GI and GII NoV in strawberry puree was more efficient from the strawberry puree compared to the two other soft red fruit types. In conclusion, results show that this methodology can be used for detection of NoV in different soft red fruits, although NoV recovery efficiencies could be influenced by (1) the NoV concentration on the soft red fruit type and (2) the tested soft red fruit type.

5.2. Introduction

Noroviruses (NoV) are recognized as one of the leading causes of gastroenteritis in people of all age groups worldwide (Koopmans and Duizer, 2004). A number of reports have described these viruses as causative agents of food- and water borne gastroenteritis outbreaks in Europe and in the USA. In 5 different European countries, 7e24% of viral (of which >90% NoV) gastroenteritis outbreaks were considered food borne (Lopman et al.,

2003b). Between 1994 and 2005, 6.6% of NoV outbreaks in the Netherlands were caused by ingestion of contaminated food (Svraka et al., 2007), while 5.2% of NoV outbreaks were caused by food borne transmission in England and Wales between 1992 and 1999 (O'Brien et al., 2000). In the USA, contaminated food was the most reported vehicle of infection as it has been estimated that 40 to 57% of all NoV outbreaks were caused by this transmission type (Fankhauser et al., 2002; Mead et al., 1999). In a number of food borne NoV outbreaks, fruits and vegetables were regarded as the causative agents. In particular, raspberries (Cotterelle et al., 2009; Falkenhorst et al., 2005; Le Guyader et al., 2009), tomatoes (Rutjes et al., 2006a; Zomer et al., 2009), and salad vegetables (Lopman et al., 2003a) were considered as the causative food vehicle. Fruits and vegetables can be contaminated before harvesting by coming into contact with (sewage) water polluted with NoV (Horman et al., 2004; van den Berg et al., 2005). Postharvest contamination can occur during processing, storage, distribution or preparation and often the foodhandler plays a crucial role (Baert et al., 2009b; Parashar et al., 1998). Although efforts have been made recently (Asanaka et al., 2005; Straub et al., 2007), there is currently no reliable culture method available to detect NoV (Duizer et al., 2004). Therefore, detection of NoV relies solely on molecular methods, and real-time RT-PCR is currently considered as the gold standard for detection of NoV in clinical, food and environmental samples (Jothikumar et al., 2005b; Park et al., 2008; Wolf et al., 2007). Compared to clinical samples, detection of NoV on food samples is hardened due to the low concentration of virus particles and the presence of substances inhibiting molecular methods used for the detection and quantification of genomic material (Rijpens and Herman, 2002; Wilson, 1997). To address this problem, a number of elution-concentration methods for extraction of viral pathogens on fresh produce have been described (Baert et al., 2008a; Butot et al., 2007; Dubois et al., 2002; Love et al., 2008). The current study describes the evaluation of a proposed NoV detection methodology consisting of (1) a NoV elution-concentration method described by Baert and colleagues (Baert et al., 2008a) and (2) a multiplex real-time RT-PCR described in chapter 2 of this PhD dissertation. The sensitivity of the method was determined on artificially contaminated frozen raspberry crumb samples, while the robustness of the method was analyzed using a number of artificially contaminated soft red fruit products.

5.3. Material & Methods

5.3.1. Soft red fruit products used for evaluation

Frozen raspberry crumb samples were kindly provided by a local food manufacturer, while a frozen forest fruit mix, fresh strawberry puree and fresh raspberries were purchased at local food stores. Frozen raspberry crumb samples were composed solely of raspberry

pieces <5 mm, while the fresh strawberry puree only contained mixed and homogenized strawberries. The frozen forest fruit mix contained strawberries, raspberries, blackberries, blueberries and black currants. Raspberries, strawberries and all fruits present in the forest fruit mix consist mainly of water and carbohydrates, but are also a rich source of bioactive compounds such as phenolics, anthocyanins, organic acids, minerals and more (Oszmianski and Wojdylo, 2009; Tosun et al., 2009). All green parts were removed from the fresh raspberries before inoculation, and the strawberry puree contained strawberries solely with no added ingredients. The different fruit types in the frozen forest fruit mix did also not contain any green parts.

5.3.2. Artificial contamination of soft red fruit products.

Soft red fruit products were artificially contaminated with GI and/or GII NoV and/or with MNV-1. For the GI and/or GII NoV inoculation, 2 stool samples containing GI.2 and GII.4 NoV, respectively, were friendly provided by the Rega Institute for Medical Research (Leuven, Belgium). Additionally, the soft red fruit samples were also artificially contaminated with diluted MNV-1 virus lysate (Baert et al., 2008b). Tenfold serial dilutions of both faecal samples and of the MNV-1 virus lysate were prepared in PBS and stored at -80 °C until use. GI and/or GII NoV were incubated overnight, while the incubation times for MNV-1 inoculation were 30 min or overnight incubation. Concentrations of the GI and GII NoV genomic copies present in the diluted stool samples and of MNV-1 in the the diluted virus lysate were determined by real-time RT-PCR.

5.3.3. Virus extraction method

The virus extraction method was performed as described by Baert and colleagues (Baert et al., 2008a). Briefly, 10 g of food product was washed with 30 ml of elution buffer (0.1 M Tris-HCl, 3% beef extract, 0.05 M glycine, pH 9.5 adjusted with 10 M NaOH) and 150 ml of Pectinex 1XL (Novozymes, Dittingen, Switzerland) on a shaking platform during 20 min in a stomacher bag with filter compartment. The filtrate was transferred to a 50 ml centrifuge tube and centrifuged (10,000 × g, 15 min, 4 °C). The pH of the supernatant was adjusted to 7.2-7.4 with 0.1 M NaOH and 6 M HCl (Sigma, Steinheim, Switzerland). Subsequently, PEG 6000 and NaCl were added to a final concentration of 10% wt/vol and 0.3 M, respectively. The samples were placed overnight on a shaking platform (4 °C). The next day the samples were centrifuged (10,000 × g, 30 min, 4 °C) and the pellet was dissolved in 1 ml of PBS. The dissolved pellet was treated with one volume of chloroform/ butanol (1:1 vol/vol) to remove inhibitory substances from the virus extract and centrifuged again (10,000 × g, 15 min, 4 °C). The aqueous phase (supernatans) was isolated and stored at -20 °C until RNA isolation.

5.3.4. RNA purification

Hundred ml of the aqueous supernatans was used for RNA purification from the extracted virus particles with an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturers' RNA Cleanup protocol (elution volume: 30 ml). All RNA purifications were stored at -20 °C until used.

5.3.5. Reverse transcription

A first pre-reaction mix consisting of 3 ml of extracted RNA and 1 ml of random hexamers (50 mM; Applied Biosystems, Foster City, CA, USA), in a final volume of 11.5 ml, was heated to 95 °C during 2 min, then cooled on ice during 2 min (thus avoiding the presence of secondary structures in the RNA and allowing the full hybridization of the RNA with the random hexamers). This first pre-reaction mix was then mixed with a second pre-reaction mix of 8.5 ml to obtain a final 20 ml RT-mastermix containing 2.5 mM random hexamers (Applied Biosystems), 25 U of Multiscribe reverse transcriptase (Applied Biosystems), 20 U of RNase inhibitor (Applied Biosystems), 5 mM MgCl2 (Applied Biosystems), 1 × PCR buffer II (10 mM Tris HCl, pH 8.3, 50 mM KCl; Applied Biosystems), 0.1 mM dNTPs (GE Healthcare; Diegem, Belgium) and isolated RNA. Reverse transcription was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) with the following temperature profile: 22 °C for 10 min, 42 °C for 15 min, 99 °C for 5 min and 5 °C for 5 min. All cDNA was stored at -20 °C.

5.3.6. Real-time PCR

A multiplex real-time PCR for simultaneous detection of GI and GII NoV and for MNV-1 was carried out as described in chapter 2 of this PhD dissertation. Briefly, the 25 ml reaction mix consisted of 5 ml template DNA, 12.5 ml of TaqMan Universal PCR Master Mix (Applied Biosystems) containing dUTP and uracyl N-glycosylase (UNG), primers and hydrolysis probes. For GI NoV detection, primers QNIF4 (500 nM), NV1LCR (900 nM) and hydrolysis probe NVGGIp (100 nM) were used, while for GII NoV detection, primers QNIF2 (500 nM), COG2R (900 nM) and hydrolysis probe QNIFS (250 nM) were used. Finally, for detection of MNV-1, primers FW-ORF1/ORF2, RV-ORF1/ ORF2 and fluorescent minor groove binding TaqMan® probe MGBORF1/ ORF2 were all used in a final concentration of 200 nM. All primers and hydrolysis probes were purchased from Eurogentec (Liège, Belgium), except the minor groove binding TaqMan® probe, which was purchased from Applied Biosystems. Real-time quantification was performed on the Lightcycler® 480II real-time PCR instrument (Roche Diagnostics, Mannheim, Germany) under the following conditions: incubation at 50 °C for 2 min to activate UNG, initial denaturation/activation at 95 °C for 10 min, followed by 50 cycles of amplification with denaturation at 95 °C for 15s and annealing and extension at

60 °C for 1min. Amplification data were collected and analysed with the Lightcycler® 480II instruments' software. In some cases, primers and hydrolysis probes for detection of MNV-1 were used as a singleplex real-time PCR assay. All real-time PCR reactions were duplicated except when mentioned. Analysis of the standard curves of the GI/GII NoV and MNV-1 multiplex assay showed PCR efficiencies of 92.9%, 84.7% and 87.0%, respectively, while the MNV-1 singleplex assay had a 86.0% PCR efficiency. Intercepts of the multiplex assay were situated at 43.3, 43.7 and 39.7, respectively, while the MNV-1 singleplex assay showed an intercept of 42.8. All R^2 values were at least 0.997.

5.3.7. Overview detection strategy

An overview of the NoV detection strategy is provided in Fig 5.1. Twenty grams of a soft red fruit product was split in 2 subsamples of each 10 g. Except for the negative control samples, both subsamples were subsequently artificially contaminated with GI and/or GII NoV and incubated at 4 °C overnight. The first subsample (10 g) was additionally spiked with 1 ml of an MNV-1 solution containing 10^6-10^7 genomic MNV-1 copies/ml, which functioned as full detection process control (MNV-1 PC). The MNV- 1 PC was incubated during 30 min at room temperature. After virus extraction, RNA cleanup and reverse transcription (RT), the recovery efficiency of the MNV-1 PC was determined in the subsample by singleplex real-time RT-PCR for detection of MNV-1 (The presence of GI/GII NoV was not analyzed in this subsample). Viral RNA of the second subsample (10 g) of the food product was extracted in parallel with the first subsample and RT of the isolated RNA was performed in duplicate. One ml of MNV-1 RNA (containing 103e104 RNA copies) was added to the RT reaction mix of 1 of the duplicate RT reactions as RT control (MNV-1 RTC). Finally, GI/GII NoV and MNV-1 copy DNA (cDNA) was detected by multiplex real-time PCR as described above. One ml (containing ca. 10^2 plasmid copies) of plasmid p20.3 containing a full MNV-1 genome (Sosnovtsev et al., 2006) was added to the real-time PCR reaction mix of the cDNA preparation without MNV-1 RTC as real-time PCR internal amplification control (MNV-1 IAC). All real-time PCR reactions were duplicated.

Thus, 2 singleplex real-time PCR reactions for detection of the MNV-1 PC were performed in the first 10 g food subsample (inoculated with the MNV-1 process control (PC)). In the second 10 g food subsample (inoculated with GI and/or GII NoV), 4 multiplex real-time PCR reactions were executed for the detection of GI and GII NoV (2 reactions with the MNV-1 RTC and 2 reactions with the MNV-1 IAC).

The recovery of GI and GII NoV and MNV-1 PC were both quantitatively and qualitatively analyzed. Quantitative analysis was performed by comparing the mean recovered number of GI/GII NoV or MNV-1 PC genomic copies with the mean inoculated number of GI/GII NoV or MNV-1 PC genomic copies. Qualitative analysis of the recovery of GI/GII NoV and

MNV-1 PC was calculated by comparison of the number of positive real-time PCR signals to the number of performed real-time PCR reactions. The quantitative and qualitative analyses were expressed respectively as the “recovery efficiency” and “recovery success rate” in Tables 5.1 and 5.2. Finally, the theoretical detection limit (100% recovery efficiency) of this method was determined at 400 genomic NoV copies per 10 g of soft red fruit food product.

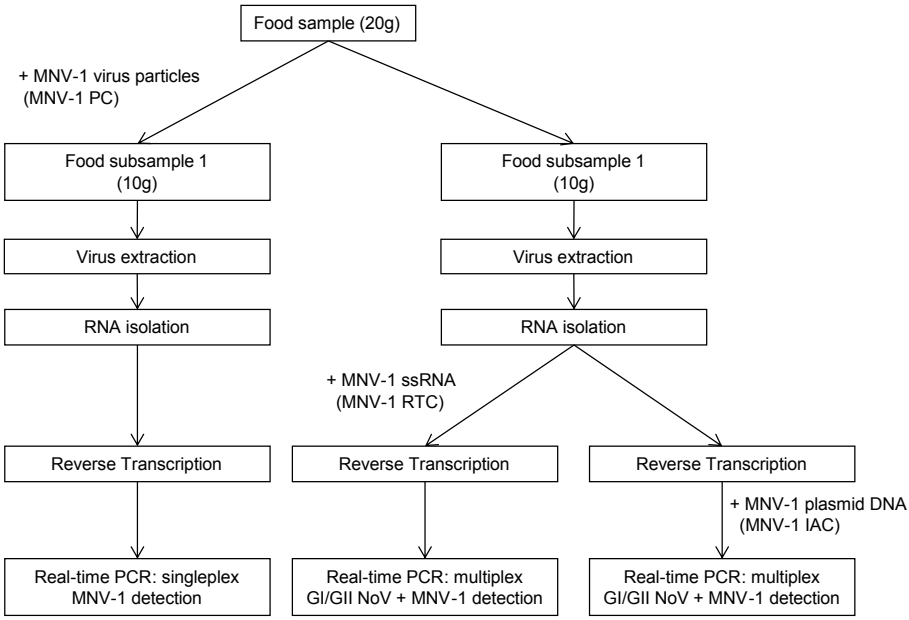

Fig. 5.1 Overview of the strategy used for detection of NoV in soft red fruit products.

5.3.8. Statistical analysis

For the quantitative analyses, a two-way analysis of variance (2- way ANOVA) was used to investigate (1) the effect of incubation time and inoculation concentration on the recovery efficiency of MNV-1 from frozen raspberry crumb and (2) the effect of the presence of different concentrations of GI and GII NoV on the recovery efficiencies of GI and GII NoV from raspberries. In addition, a one way analysis of variance (1-way ANOVA) was used to investigate the effect of the GI or GII NoV inoculation concentrations on the recovery efficiencies of GI or GII NoV, respectively. As the assumptions for a 1-way ANOVA analysis were not fulfilled for the data describing the influence of the soft red fruit type (deep frozen forest fruit mix, fresh raspberries or strawberry puree) on the recovery efficiencies of GI and GII NoV and MNV-1, a nonparametric Kruskale Wallis test (KW test) was applied. All

statistical analyses were performed using the statistical package R (version 2.10.1; R Foundation for Statistical Computing).

5.4. Results

5.4.1. Effect of the incubation time and inoculum level on the MNV-1 recovery

The effect of the incubation time and inoculum level on the MNV-1 recovery efficiency was analyzed by inoculating 2 different levels of MNV-1 on not GI/GII NoV inoculated soft red fruit samples during 2 incubation periods: thirty minutes or overnight (both at 4 °C). In detail, high (2.66×10^8 genomic copies) and lower (2.66×10^6 genomic copies) levels of MNV-1 were inoculated on 10 g of frozen raspberry crumb. Since no GI or GII NoV were inoculated, the detection strategy presented in Fig. 5.1 was not applied. Every combination of incubation time/inoculum level was triplicated, while real-time PCR reactions were not duplicated. Additionally, two not inoculated samples were analyzed as negative controls. The recovery efficiency of MNV-1 from raspberries was not significantly influenced by either the MNV-1inoculum levels (2-way ANOVA; $p = 0.8$) or the incubation time (2-way ANOVA; $p = 0.36$). In detail, high and low level MNV-1 inoculum levels were recovered with mean efficiencies of 28.75 ± 14.40% and 16.00 ± 14.58%, respectively. MNV-1 could be recovered with mean efficiencies of 19.30 ± 15.98% and 25.45± 17.87% after a thirty minute or overnight incubation period, respectively. Since no significant differences were found, an MNV-1 concentration resembling the lower MNV-1 inoculum level (ca. 10^6 MNV-1 genomic copies) in combination with a 30 min incubation time was chosen as MNV-1 PC in the NoV detection strategy (Fig. 5.1).

5.4.2. Determination GI and GII NoV recovery efficiency

To examine the recovery of GI and GII NoV from soft red fruits, 2 different inoculum levels of GI and/or GII NoV were spiked onto both frozen raspberry crumb subsamples (each 10 g) as described in the detection strategy (Fig. 5.1) and incubated over night at 4 °C, resulting in 8 different inoculation(s) (combinations) (Table 5.1). Every inoculation (combination) was performed in duplicate. Additionally, two not GI or GII NoV inoculated samples were analyzed as negative control samples.

Quantitative analysis showed that the recovery efficiencies of GI and GII NoV solely from the frozen raspberry crumb samples were influenced significantly by their inoculum level (1-way ANOVA; $p = 0.002$ and $p = 0.0001$, respectively). In addition, the presence of GI or GII NoV did not significantly influence the recovery of GII or GI NoV (2-way ANOVA; $p = 0.65$ and $p = 0.36$, respectively). An overview of the obtained results is shown in Table 5.1.

In detail, GI NoV genomic copies were recovered with a mean efficiency of 28.44 ± 3.09 % (high inoculum level) and 6.41 ± 4.62 % (low inoculum level) without the presence of GII

NoV. In combination with the high and low level GII NoV inoculum, the high level GI NoV inoculum was recovered with mean efficiencies of 22.05± 8.31 % and 19.26 ± 4.64 %, respectively. Similarly, 9.71% and 11.79 ± 7.30 % of the low level GI NoV inoculum could be recovered in the presence of the high and low level GII NoV inoculum, respectively.

GII NoV genomic copies were recovered with mean efficiencies of 12.79 ± 2.93 % (high inoculum level) and 5.70 ± 1.47 % (low inoculum level) without the presence of GI NoV. In combination with the high and low level GI NoV inoculum, the high level GII NoV inoculum was recovered with mean efficiencies of 15.18 ± 5.39 % and 12.28 ± 1.09 %, respectively. Similarly, 2.93 ± 0.93 % and 7.00 ± 1.38 % of the low level GI NoV inoculum could be recovered in the presence of the high and low level GI NoV inoculum, respectively.

Table 5.1: influence of NoV inoculum level on NoV extraction efficiencies from 10 grams of artificially contaminated deepfrozen raspberry crumb samples. Every inoculation (combination) was duplicated.

Inoculum	GI NoV Inoculum level (genomic copies/10g)	GII NoV Inoculum level (genomic copies/10g)	Recovery efficiency[a] GI NoV ± stdev (success rate)[b]	Recovery efficiency[a] GII NoV ± stdev (success rate)[b]	Recovery efficiency[a] MNV-1 PC ± stdev (success rate)[b]
GI NoV	1.47×10^7	/[c]	28.44 ± 3.09 % (4/8)	Negative	12.79 ± 2.10 % (4/4)
	1.95×10^5	/	6.41 ± 4.64 % (8/8)	Negative	14.34 ± 1.94 % (4/4)
GII NoV	/	7.09×10^7	Negative	12.79 ± 2.93 % (8/8)	12.40 ± 0.42 % (4/4)
	/	2.32×10^6	Negative	5.70 ± 1.47 % (8/8)	13.63 ± 2.04 % (4/4)
GI + GII NoV	1.47×10^7	7.09×10^7	22.05 ± 8.31 % (8/8)	15.18 ± 5.39 % (8/8)	15.99 ± 5.84 % (4/4)
	1.47×10^7	2.32×10^6	19.26 ± 4.64 % (8/8)	2.93 ± 0.93 % (8/8)	14.18 ± 6.32 % (4/4)
	1.95×10^5	7.09×10^7	9.71 % (1/8)	12.28 ± 1.09 % (7/8)	20.49 ± 1.29 % (4/4)
	1.95×10^5	2.32×10^6	11.79 ± 7.30 % (5/8)	7.00 ± 1.38 % (5/8)	19.61 ± 1.71 % (4/4)
Negative control	/	/	Negative	Negative	15.69 ± 7.06 % (4/4)

[a] ((Mean number GI/GII NoV or MNV-1 genomic copies recovered from 10g of inoculated fruit sample)/(Number GI/GII NoV or MNV-1 genomic copies inoculated on 10g of fruit sample)) × 100 %

[b] # positive real-time PCR reactions / # performed real-time PCR reactions

[c] Not added

No inhibition of the reverse transcription reactions or real-time PCR was noticed for any of the samples. The MNV-1 RTC and MNV-1 IAC were detected without matrix at Ct values of 23.61 and 30.34, respectively and were in all samples detected at Ct values of 24.06 ± 0.32 and 30.11 ± 0.41. The MNV-1 PC could be recovered in all samples with a mean efficiency of 15.46 ± 4.00 %.

Qualitative analysis showed that the high level GI NoV inoculum could be recovered with success rates of 4/8, 8/8 and 8/8 when solely inoculated and in combination with a low and high level GII NoV inoculum, respectively. In contrast, while the low level GI NoV inoculum

could be recovered solely with a success rate of 8/8, success rates of 5/8 and 1/8 were noticed in combination with the low and high level GII NoV inoculum. The high level GII NoV inoculum could be recovered with success rates of 8/8,7/8 and 8/8 when solely inoculated and in combination with a low and high level GI NoV inoculum respectively. Finally, while the low level GII NoV inoculum could be recovered solely with a success rate of 8/8, success rates of 5/8 and 8/8 were observed in combination with a high and low level GI NoV inoculum, respectively.

5.4.3. Evaluation NoV detection methodology on various soft red fruit products

To examine the robustness of the NoV detection methodology on a range of soft red fruit products, low levels of GI and GII NoV were inoculated onto both 10 g soft red fruit subsamples (deep frozen forest fruit mix, fresh raspberries or strawberry puree) as described in the detection strategy (Fig. 5.1). Inoculation(s) (combinations) were not duplicated. For each soft red fruit type, a not GI or GII NoV inoculated 10 g sample was analyzed as negative control (Table 5.2).

Quantitative analysis showed that the recovery efficiency of GI NoV and MNV-1 inocula differed significantly according to the fruit type tested (KW test; $p = 0.037$ for GI; $p = 0.021$ for MNV-1). No such significant influences were noticed for the recovery of the GII NoV inoculum (KW test; $p = 0.21$).

In detail, the GI NoV inoculum was recovered in the deepfrozen forest fruit mix with mean efficiencies of 7.42 ± 2.65% and 13.47 ± 7.72%, respectively solely inoculated and in combination with the GII NoV inoculum. In contrast, the GII NoV inoculum could only be recovered in combination with the GI NoV inoculum with a mean efficiency of 20.68 ± 18.27% in the deepfrozen forest fruit mix, while no recovery was noticed when inoculated solely (it should also be noted that the MNV-1 PC was in this sample recovered with a low efficiency (7.78%)).

In the fresh raspberries, the GI and GII NoV inocula were recovered with mean efficiencies of 21.50 ± 6.74% and 35.20 ± 31.54% when inoculated solely. Recovery of the combined GI and GII NoV inoculum was not possible from this fruit product.

In the fresh strawberry puree, the GI NoV inoculum was recovered with mean efficiencies of 51.13 ± 38.24% and 61.06 ± 40.11%, respectively solely inoculated and in combination with the GII NoV inoculum. Similarly, the GII NoV inoculum could be recovered with mean efficiencies of 47.72 ± 25.43% and 25.26 ± 19.08%, respectively solely inoculated and in combination with the GI NoV inoculum.

Table 5.2 Influence of soft red fruit type on NoV extraction efficiencies from artificially contaminated soft red fruit products. Inoculation(s) (combinations) were not duplicated.

Food type	GI NoV Inoculum level (genomic copies/10g)	GII NoV Inoculum level (genomic copies/10g)	Recovery efficiency[a] GI NoV ± stdev (recovery success rate)[b]	Recovery efficiency[a] GII NoV ± stdev (recovery success rate)[b]	Recovery efficiency[a] MNV-1 PC (recovery success rate)[b]
Deepfrozen forest fruit mix	3.99×10^4	/[c]	7.42 ± 2.65 % (2/4)		23.65% (2/2)
	/	9.63×10^4		Negative (0/4)	7.78% (2/2)
	3.99×10^4	9.63×10^4	13.47 ± 7.72 % (4/4)	20.68 ± 18.27 % (4/4)	28.78% (2/2)
	/	/	Negative	Negative	32.78% (2/2)
Fresh raspberries	3.99×10^4	/	21.50 ± 6.74 % (4/4)		8.29% (2/2)
	/	9.63×10^4		35.20 ± 31.54 % (2/4)	25.76% (2/2)
	3.99×10^4	9.63×10^4	Negative (0/4)	Negative (0/4)	21.10% (2/2)
	/	/	Negative	Negative	12.87% (2/2)
Fresh strawberry puree	3.99×10^4	/	51.13 ± 38.24 % (4/4)		52.05% (2/2)
	/	9.63×10^4		47.72 ± 25.43 % (4/4)	39.64% (2/2)
	3.99×10^4	9.63×10^4	61.06 ± 40.11 % (4/4)	25.26 ± 19.08 % (3/4)	75.65% (2/2)
	/	/	Negative	Negative	42.23% (2/2)

[a] ((Mean number GI/GII NoV or MNV-1 genomic copies recovered from 10g of inoculated fruit sample)/(Number GI/GII NoV or MNV-1 genomic copies inoculated on 10g of fruit sample)) × 100 %

[b] # positive real-time PCR reactions / # performed real-time PCR reactions

[c] Not added

The mean recovery efficiencies of the MNV-1 PC in all deepfrozen forest fruit, fresh raspberry and fresh strawberry puree samples were 17.01 ± 7.89%, 23.25 ± 10.97% and 52.39 ± 16.40%, respectively. No inhibition of the real-time PCR was noticed for any of the samples, while a somewhat less efficient could be noticed for the reverse transcription: the MNV-1 RTC and MNV-1 IAC were detected without matrix at respective Ct values of 27.53 and 28.89 and were in all samples detected at Ct values of 29.24 ± 0.70 and 29.01 ± 0.39.

Qualitative analysis showed that, while recovery success rates of 2/4 and 0/4 were noticed in the deepfrozen forest fruit mix when GI and GII NoV were solely inoculated, a recovery success rate of 4/4 was noticed for both GI and GII NoV inocula when simultaneously inoculated on the deepfrozen forest fruit mix. While GI and GII NoV could be recovered with success rates of respectively 4/4 and 2/4 when inoculated solely on fresh raspberries, no recovery was noticed when GI and GII NoV were inoculated simultaneously (recovery success rates: 0/4). In addition, a recovery success rate of 4/4 was noticed in the fresh strawberry puree for GI when solely inoculated and or simultaneously with the GII NoV inoculum. For the GII NoV inoculum, recovery success rates of 4/4 and 3/4 were noticed in the fresh strawberry puree, respectively solely inoculated and in combination with the GI

NoV inoculum. Finally, recovery of the MNV-1 PC was successful in all soft red fruit types (12/12).

5.5. Discussion

In the presented study, a methodological approach for the detection and quantification of noroviruses (NoV) in soft red fruit products is proposed and evaluated. The proposed NoV detection method is the combination of (1) a viral RNA extraction method developed by Baert et al. (2008a), (2) an RNA purification method and (3) a multiplex real-time RTPCR assay for simultaneous detection of genogroup I (GI) and II (GII) NoV and the murine norovirus-1 (MNV-1) as described in chapter 2 of this PhD dissertation. The described NoV detection strategy was based on the use of controls at different steps throughout the procedure that are considered critical for correct quantification: the reverse transcription of the extracted and isolated RNA and the real-time PCR reaction. Genomic MNV-1 RNA was used as reverse transcription control (MNV-1 RTC) and a plasmid containing a full genome of MNV-1 was used as real-time PCR internal amplification control (MNV-1 IAC). In parallel, the full NoV detection procedure was controlled using MNV-1 virus particles as process control (MNV-1 PC). Costafreda and colleagues (2006) have described a similar approach for detection of (HAV) in shellfish. However, the latter study differed from the current study in two points. First of all, the protocol only included a reverse transcription control (RTC) and a full detection procedure process control (PC) with the RTC consisting of a ssRNA fragment containing the primer-probe binding sites of the developed HAV real-time RT-PCR assay in the study. Secondly, a genetically modified mengovirus (vMC_0, a HAV surrogate virus) was added to the artificially contaminated sample as PC.

In contrast to other studies (Escobar-Herrera et al., 2006; Trujillo et al., 2006b), the MNV-1 RTC in the current study was not based on synthetic run-off RNA transcripts, since such short nucleic acid fragments can easily cause laboratory contamination, leading to false-positive results as described in chapter 3 of this PhD dissertation. The use of MNV-1 as process control in NoV detection protocols has been proposed due to its genetic similarities towards the NoV genome (Baert et al., 2008b; Wobus et al., 2006). Although a number of methods have been published for detection of NoV in fruits and vegetables, no standardized method has been approved yet. Therefore, most labs are restricted to their in-house developed methods. A possible reason for this lack of standardization is the limited attention spent on the evaluation and validation of such existing methods. However, recent efforts have been made within various EU projects, between reference laboratories and within the European Committee for Standardization/Technical Committee/Working Group 6/Task Group 4 on virus detection in foods (the CEN/TC275/WG6/TAG4 working group), to stimulate the acceptance of a standardized method (Croci et al., 2008; Rodriguez-Lazaro et

al., 2007). The sensitivity of the NoV detection method was investigated by analysis of the influence of (1) the GI and GII NoV and MNV-1 inoculum levels and (2) the inoculum incubation time of MNV-1 on the recovery efficiencies and recovery success rates. In general, GI and GII NoV could be recovered in frozen raspberry crumb samples with mean efficiencies varying between 6 and 28% and 3 to 15%, respectively. A similar NoV elution-concentration protocol designed by Butot et al. (Butot et al., 2007) recovered 2160 RT-PCRU of GI.4 NoV per 60 g of food product with efficiencies of 1.7%, 2.6%, 17.9% and 19.6% in fresh strawberries, frozen raspberries, frozen blueberries and fresh raspberries, respectively. In a recent study, GI and GII NoV were extracted from artificially contaminated strawberries by combining a similar NoV elution-concentration method with an immunomagnetic separation technique (Park et al., 2008). In the latter study, $4 \times 10^3 – 10^4$ GI and GII NoV RT-PCRU could be recovered with efficiencies of 29.50% and 14.14%, respectively. A recent study comparing different aspects of the NoV elution - PEG concentration method (using conventional RT-PCR) showed that 85% recovery of 4×10^4 GII.4 NoV RT-PCRU from fresh strawberries was possible when combining a 3% beef extraction buffer as elution buffer with 8% (w/v) PEG8000 precipitation (Kim et al., 2008a). Finally, Cheong et al. (2009a) obtained 3.9% to 50% recoveries when extracting $4.8 \times 10^0 – 10^3$ GII NoV RT-PCRU from 5 g of strawberries by comparing different elution buffers in a similar elution-concentration detection protocol.

Quantitative and qualitative results from the presented study showed that, although the inoculum level had a significant influence on the recovery efficiency of GI and GII NoV in frozen raspberry crumb samples, no significant effect of the presence of GI and GII NoV was noticed on the recovery of GII and GI NoV, respectively. Additionally, no significant influence of the inoculum time and inoculum level was noticed on the recovery efficiency of MNV-1. In concordance with these results, a recent analysis of naturally contaminated shellfish samples artificially contaminated with a genetically modified mengovirus process control showed that there were no differences in extraction efficiencies of the process control when GI and GII NoV were separate or simultaneously present (Le Guyader et al., 2009). The influence of the NoV concentration NoV on the recovery efficiency has been investigated by several authors, yet no consensus could be found. Fumian et al. (2009) found that high levels (pure and 10% PBS diluted fecal samples) of GI NoV on lettuce caused lower extraction efficiencies (approximately 1 log) compared to lower levels (1% and 0.1% PBS diluted fecal samples). Such results were not noticed in our study. However, the authors did not include an inhibition control in the detection method, hence possible inhibition of the reverse transcription reaction or real-time PCR could not be excluded. In contrast, a recent study showed that the recovery of NoV in lettuce was more successful at higher concentrations, both in small and high volumes of tested food samples (Cheong et

al., 2009a). Finally, the robustness of the proposed NoV detection methodology was investigating by inoculating low levels (approximately 10^4 genomic copies/10 g soft red fruit product) of GI and/or GII NoV on various soft red fruit products.

In general, a significant influence of the soft red fruit product type was noticeable on the protocol. Extraction of GI NoV and of the MNV-1 PC was more efficient in fresh strawberry puree samples compared to deepfrozen forest fruit samples or fresh raspberries, while no such effect was noticed for GII NoV. The effect of different food matrices on the quantification of NoV in food products has only been investigated by a limited number of authors. A recent study combining carbohydrate-coated magnetic beads with conventional RT-PCR showed that the recovery of NoV from lettuce and green onions had a higher success rate compared to the recovery of NoV from fresh strawberries (Morton et al., 2009). Results obtained in the current study were in contrast to a very similar extraction method developed by Dubois et al. (2002). The latter study showed a tenfold less efficient recovery of the NoV elution-concentration method when tested on mashed strawberries compared to frozen raspberries and fresh strawberries.

5.6. Conclusions

In conclusion, a NoV detection methodology has been successfully evaluated, consisting of (1) a described NoV virus extraction method (Baert et al., 2008a), (2) an RNA purification method and (3) real-time RT-PCR assays for detection of GI and GII NoV and/or MNV-1 as described in chapter 2 of this PhD dissertation. Additionally, a quantitative NoV detection strategy was proposed in which the murine norovirus-1 was successfully included as full detection procedure process control, reverse transcription control and real-time PCR internal amplification control. Results showed that the proposed NoV detection method is able to detect high and low NoV concentrations on a range of soft red fruit products. This NoV detection methodology therefore provides a reliable means for detection of human noroviruses in soft red fruit products. Moreover, this detection methodology and strategy were applied in chapter 6 for the screening of 75 fruit samples for NoV presence.

5.7. Acknowledgements

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Chapter 6

SCREENING OF FRUIT PRODUCTS FOR NOROVIRUS AND THE DIFFICULTY OF INTERPRETING POSITIVE PCR RESULTS

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6. CHAPTER 6: SCREENING OF FRUIT PRODUCTS FOR NOROVIRUS AND THE DIFFICULTY OF INTERPRETING POSITIVE PCR RESULTS.

6.1. Abstract

Despite recent norovirus (NoV) outbreaks related to consumption of fruit products, little is known regarding the NoV load on these food products. Therefore, a total of 75 fruit products (raspberries, strawberries, cherry tomatoes and fruit salads) were screened for NoV presence using an evaluated in-house NoV detection methodology consisting of a NoV extraction method and a multiplex real-time reverse transcriptase (RT-) PCR assay. Additionally, the fruit samples were screened for bacterial pathogens and bacterial hygiene indicators.

Results of the NoV screening showed that 18 out of 74 samples tested positive for GI and/or GII NoV genomic material despite a good bacteriological quality (a single raspberry sample showed inhibition of the real-time RT-PCR and no results could thus be drawn from this sample). The recovery of the murine norovirus 1 (MNV-1) virus particles acting as a process control was successful in 31/74 samples with a mean recovery efficiency of 11.32 ± 6.08 %.

The level of detected NoV genomic copies concentrations ranged between 2.5 and 5.0 logs per 10 grams of fruit sample. NoV GI and/or GII were found in 4/9, 7/30, 6/20 and 1/15 of the tested raspberries, cherry tomatoes, strawberries and fruit salad samples, respectively. However, confirmation of the positive real-time PCR results by sequencing genotyping regions in the NoV genome was not possible. Due to the nature of the used method (real-time RT-PCR) for detection of genomic material (RNA), no differentiation was possible between infectious and non-infectious viral particles. No Nov outbreaks were reported related to the tested fruit product types during the screening period, which troubles the conclusion whether or not these unexpected high number of NoV positive results obtained should be perceived as a public health threat. These results however, may indicate a prior NoV contamination of the tested food samples throughout the fresh produce chain

6.2. Introduction

Due to the beneficial effects of fruits and vegetables on the consumer's health, efforts have been made recently to promote the inclusion of these food products into daily diets, and a minimum daily consumption of 400 gram is recommended. (Joffe and Robertson, 2001; Van Duyn and Pivonka, 2000). However, the increased consumption of fruits and vegetables has also been linked to an increased number of food borne outbreaks caused by these food types (Hedberg et al., 1994). In particular, a number of acute bacterial and non-bacterial gastroenteritis food borne outbreaks have been linked to consumption of fresh fruit products, and have been summarized in various review articles (Rutjes et al., 2006a; Sivapalasingam et al., 2004).

Food borne outbreaks related to fruit products are probably mainly caused via two transmission routes. The fruit product can be contaminated by pre-harvest manipulations such as the use of contaminated irrigation water (Hernandez et al., 1997; Mukherjee et al., 2004) and a second possible cause of these food borne outbreaks is by (post-) harvest contamination of the fruit product. In the latter contamination type often an infected food handler or the use of contaminated equipment/process water are involved (Beuchat, 1996; Sair et al., 2002).

To investigate whether these transmission routes might introduce NoV in the food chain, data are needed regarding NoV prevalence on fresh produce.

The goal of the current study was therefore twofold. First of all, the presence of noroviruses (NoV) was determined on a total of 75 raspberry, cherry tomato, strawberry and mixed fruit salad samples using a developed and evaluated in-house NoV detection methodology for fruit products described in chapter 5 of this PhD dissertation. The use of (genomic material of) MNV-1, a cultivable NoV surrogate virus, was applied as control on different crucial steps in the NoV analysis. Secondly, the presence of three bacterial pathogens (*E. coli* 0157:H7, *Salmonella*, and *Listeria spp./monocytogenes*) and enumerations of *Enterobacteriaceae* and *E. coli* were analyzed in all tested fruit samples.

6.3. Materials and Methods

6.3.1. Overview analyzed samples.

A total of 75 fruit samples that might pose a risk for NoV contamination (raspberries, cherry tomatoes, strawberries and fruit salads) was analyzed for norovirus (NoV) and bacteriological pathogens and indicators. For raspberries, two lots (originating from Serbia and Poland) containing five samples originating from five different farmers were tested. For cherry tomatoes and strawberries, respectively three and two lots (originating from Spain) containing each ten samples were analyzed. Finally, fifteen mixed fruit salads (prepared in Belgium) were examined. All samples were friendly provided by local manufacturers and

distributors. Bacteriological analysis was performed within 24 hour of arrival of the samples, while aliquots of the samples were stored at 4°C for NoV analysis (all samples were analyzed for NoV within a maximum of 48 hours).

6.3.2. Norovirus analysis

NoV analysis was performed exactly as described in chapter 5 of this PhD dissertation. Briefly, an alkaline elution – PEG precipitation was used for virus extraction from the 10g fruit samples, the Qiagen RNeasy minikit (RNA Cleanup protocol) was used for extraction of the genomic RNA from the virus capsids and the purified RNA was detected by a multiplex real-time RT-PCR assay described in chapter 2.

The used NoV detection strategy including the use of MNV-1 as process control (MNV-1 PC), reverse transcription control (MNV-1 RTC) and real-time PCR internal amplification control (MNV-1 IAC) was described in chapter 5 as well (Fig. 5.1).

6.3.3. Bacteriological analysis

6.3.3.1. Enterobacteriaceae/Escherichia coli enumeration.

For quantitative Enterobacteriaceae and *Escherichia coli* enumerations, 25 g of the fruit sample was homogenized in 225 ml of buffered pepton water (BPW; BioMérieux, Marcy-l'Etoile, France). Subsequently, 1.0 ml of this primary dilution of the fruit samples was analyzed using the TEMPO BC® (*Enterobacteriaceae* enumeration) or TEMPO EC® (*Escherichia coli* enumeration) automated MPN methods (BioMérieux), according to the manufacturer's instructions.

6.3.3.2. Listeria spp./monocytogenes.

For detection of *Listeria* spp./*monocytogenes*, 25 g of the soft red fruit sample was diluted in 225 ml of Half-Fraser Broth (BioMérieux) and subsequently homogenized using the Seward Laboratory blender 400 (UAC House). After 20-26 hours of incubation at 30°C, 1 ml of this primary enrichment culture was transferred to a 10 ml Fraser Broth tube (Biomérieux) and subsequently 20-26 hours incubated at 30°C. Analysis of the second enrichment culture was performed using the VIDAS LIS® method (BioMérieux), according to the manufacturer's instructions.

6.3.3.3. Salmonella spp.

The presence of *Salmonella* spp. was analyzed using the iQ-Check™ *Salmonella* II kit (Bio-Rad, Nazareth Eke, Belgium) as described by the manufacturer. Briefly, 25 g of the fruit sample was diluted in 225 ml of BPW (BioMérieux) and homogenized using the Seward Laboratory blender 400 (UAC House). After 8 hours incubation at 37°C, 1 ml of the enriched

sample was centrifuged (10000 × g, 10 min, room temperature) and the supernatant was discarded. DNA was extracted by adding 200 µl of the lysis reagent followed by a short vortexing step at room temperature. Subsequently, the samples were heated for 10 min at 95 to 100°C and after a centrifugation step (10000 × g, 5 min, room temperature), 5 µl of the obtained DNA suspension was used for the amplification reaction (45 µl of PCR mixture). Real-time quantification was performed on the SDS 7300 real-time PCR instrument (Applied Biosystems) using following temperature protocol: 50 °C for 2 min, initial denaturation/activation at 95 °C for 10 min, followed by 50 cycles of amplification with denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s and extension for 30 s at 72°C. Amplification data were collected and analyzed with the SDS 7300 instruments' software.

6.3.3.4. E. coli 0157:H7.

For detection of *E. coli* 0157:H7, 25 g of the fruit sample was diluted in 225 ml of BPW (BioMérieux) and homogenized using the Seward Laboratory blender 400 (UAC House). After 8 hour incubation at 41°C, the presence of *E. coli 0157:H7* was analyzed using the iQ-Check™ *E. coli 0157:H7* kit (Bio-Rad) as described by the manufacturer. Briefly, 200 µl of the complete lysis reagent was added to 100 µl of the enriched samples followed by a short vortexing step at room temperature. Subsequently, the samples were heated for 10 min at 95 to 100°C and after a centrifugation step (10000 × g, 5 min, room temperature), 5 µl of the obtained DNA suspension was used for the amplification reaction (45 µl of PCR mixture). Real-time quantification was performed on the SDS 7300 real-time PCR instrument (Applied Biosystems) under the following conditions: initial denaturation/activation at 95 °C for 10 min, followed by 50 cycles of amplification with denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and extension for 30 s at 72°C. Amplification data were collected and analyzed with the SDS 7300 instruments' software.

6.4. Results

6.4.1. MNV-1 Controls.

The recoveries of the MNV-1 PC, MNV-1 RTC and MNV-1 IAC were both quantitatively ("recovery efficiency") and qualitatively (" recovery success rate") analyzed (Table 6.1). Qualitative analysis showed that the recovery of the MNV-1 PC was dependent of the fruit type tested: while recovery success rates of 8/10 and 19/20 were noticed in respectively raspberries and strawberries, recovery success rates of 2/30 and 2/15 were noticed in cherry tomatoes and mixed fruit salads, respectively. Quantitative analysis showed that the mean recovery efficiency of the successfully recovered MNV-1 PCs was similar in all tested fruit types as mean recovery efficiencies ranged between 8.38 ± 1.18 % and 12.94 ± 9.33 %.

Qualitative analysis of the recovery of the MNV-1 RTC and MNV-1 IAC showed that recovery success rates of 30/30, 20/20 and 15/15 were obtained in respectively cherry tomatoes, strawberries and fruit salads, while a recovery success rate of 9/10 was noticed in raspberries. Additionally, quantitative analysis of the recovery of the MNV-1 RTC and MNV-1 IAC showed the MNV-1 RTC was recovered with mean recovery efficiencies ranging between 46.17 ± 17.70 % and 63.81 ± 15.72 % , while the MNV-1 IAC was recovered with mean recovery efficiencies ranging between 100.93 ± 9.55 % and 119.32 ± 28.32 %. The rather low recovery of the MNV-1 RTC indicated limited inhibition of the reverse transcription step in some samples.

Table 6.1 Quantitative and qualitative analysis of the performance of MNV-1 as process control (MNV-1 PC), reverse transcription control (MNV-1 RTC) and real-time PCR internal amplification control (MNV-1 IAC).

Fruit type	N	MNV-1 PC		MNV-1 RTC	MNV-1 IAC
		Mean recovery efficiency[a] ± stdev (Recovery success rate)[b]	Recovery efficiency range	Recovery efficiency[a] ± stdev (Recovery success rate)[b]	Recovery efficiency[a] ± stdev (Recovery success rate)[b]
Raspberries	10	12.94 ± 9.33 % (8/10)	2.79 % - 27.27 %	46.17 ± 17.70 % (9/10)	100.93 ± 9.55 % (9/10)
Cherry tomatoes	30	11.17 ± 8.22 % (2/30)	2.57 % - 19.78 %	56.13 ± 12.31 % (30/30)	117.76 ± 9.22 % (30/30)
Strawberries	20	12.80 ± 5.60 % (19/20)	5.35 % - 19.68 %	46.54 ± 29.75 % (20/20)	114.27 ± 19.03 % (20/20)
Mixed fruit salad	15	8.39 ± 1.18 % (2/15)	7.56 % - 9.23 %	63.81 ± 15.72 % (15/15)	119.32 ± 28.32 % (15/15)
Total	75	11.32 ± 6.08 % (31/75)	2.57 % - 27.27 %	53.16 ± 18.87 % (74/75)	114.75 ± 15.70 % (74/75)

[a] Recovery efficiency: (Mean concentration MNV-1 genomic/RNA/plasmid copies inoculated) / (mean concentration MNV-1 genomic/RNA/plasmid copies recovered)) × 100 %.

[b] Recovery success rate: (# samples per fruit type with successful recovery of the MNV-1 PC/RTC/IAC) / (# samples per fruit type).

6.4.2. NoV analysis

6.4.2.1. Raspberries.

Four out of 10 raspberry samples tested positive for GI and/or GII NoV with genomic NoV concentrations in the positive samples ranging between 2.45 and 3.70 logs per 10g of raspberry sample. However, only 1 out of 4 real-time RT-PCR reactions was positive for GI or GII NoV genomic material in these samples, except for 1 sample (Table 6.2). It should be noted that in one NoV positive sample the MNV-1 PC could not be recovered, although the MNV-1 RTC and MNV-1 IAC did not indicate inhibition of either the RT step nor real-time PCR in this sample. Inhibition of all real-time RT-PCR reactions was observed in another of the samples, therefore, no conclusions could be drawn for this sample regarding the presence/absence of NoV genomic copies.

6.4.2.2. Cherry tomatoes.

Seven out of 30 cherry tomato samples tested positive for GI and/or GII NoV. Detected genomic NoV concentrations were generally higher compared to other soft red fruit samples and ranged between 3.91 and 5.04 logs per 10 g of cherry tomato sample. However, only 1 out of 4 real-time RT-PCR reactions was positive for GI and/or GII NoV in these samples, except for a single sample (Table 6.2). It should be noted that the MNV-1 PC could be recovered in only one positive sample, although the MNV-1 RTC and MNV-1 IAC did not indicate inhibition of either the RT step nor real-time PCR in all samples. A remark regarding the real-time PCR results of the cherry tomato samples is that a higher background fluorescence was noticed in the GI NoV and GII NoV assays within the multiplex real-time PCR assay in comparison to other fruit types. While this lead to a shifted standard curve and thus to corresponding higher Ct values for positive samples, this did not interfere with the quantitative properties of the real-time PCR assay.

6.4.2.3. Strawberries.

Six out of 20 strawberry samples tested positive for GI and/or GII NoV with concentrations in the positive samples ranging between 2.29 and 4.10 genomic NoV copies per 10g of strawberry sample (Table 6.2). However, only 1 out of 4 real-time RT-PCR reactions was positive for GI and/or GII NoV in all positive samples. It should be noted that in the single NoV positive sample where the MNV-1 PC could not be recovered, a low recovery efficiency (21.27 %) of the MNV-1 RTC was noticed, suggesting a possible inhibition of the reverse transcription.

6.4.2.4. Fruit salad samples.

A single fruit salad sample out of 15 tested samples tested positive for GII NoV, while no GI NoV were detected (Table 6.2). A genomic NoV concentration of 4.64 logs was observed in the positive sample with 2 out of 4 real-time RT-PCR reactions being positive. A remark regarding the real-time PCR results of the fruit salad samples, similar to the cherry tomato results, is that a higher background fluorescence was noticed in the GII NoV assay within the multiplex real-time PCR assay in comparison to other fruit types. While this lead to a shifted standard curve and thus to corresponding higher Ct values for positive samples, this did not interfere with the quantitative properties of the real-time PCR assay.

Table 6.2 Overview NoV genomic presence on tested fruit samples.

	Sample name	GI NoV presence[a] (Ct value(s))	GII NoV presence[a] (Ct value(s))	MNV-1 PC	MNV-1 RTC	MNV-1 IAC
Raspberries (n=10)	RB P03 20090421	nd[b]	3.05 (39.98)	15.35%	66.29%	104.58%
	RB P05 20090421	2.61 (40.81)	3.70 (37.66)	4.01%	54.56%	104.58%
	RB S01 20090421	2.45 (41.24; 41.47)	3.60 (38.03)	nr[c]	36.71%	93.57%
	RB S02 20090421	3.21 (38.74)	nd	8.73%	27.99%	81.14%
Cherry tomatoes (n=30)	CT 07 20090423	nd	3.91 (38.66)	nr	60.88%	114.08%
	CT 09 20090423	4.11 (40.67)	nd	nr	75.92%	123.20%
	CT 10 20090423	4.33 (39.98)	nd	nr	47.22%	126.12%
	CT 01 20090429	4.08 (38.54; 38.30)	nd	nr	39.35%	113.32%
	CT 04 20090429	nd	4.19 (41.31)	nr	59.22%	111.47%
	CT 06 20090429	4.07 (37.39; 39.53)	5.04 (38.38)	nr	62.84%	132.30%
	CT 03 20090513	4.38 (39.38)	4.67 (37.15)	16.98%	62.38%	122.60%
Strawberries (n=20)	SB 01 20090506	4.10 (39.27)	3.28 (39.75)	9.02%	101.61%	122.96%
	SB 04 20090506	nd	3.05 (40.58)	8.09%	66.44%	132.94%
	SB 06 20090506	nd	3.77 (38.01)	12.70%	58.16%	123.37%
	SB 03 20090520	2.29 (41.97)	nd	nr	21.27%	100.42%
	SB 04 20090520	2.86 (40.02)	nd	13.88%	12.45%	93.90%
	SB 07 20090520	3.40 (38.20)	nd	12.54%	58.79%	108.85%
Fruit salads (n=15)	FS 01 20090504	nd	4.64 (40.92; 40.19)	7.56%	75.67%	92.92%

[a] # of detected genomic NoV copies per 10 g fruit product sample are expressed in log scale. [b] nd: not detected. [c] nr: not recovered

6.4.3. Bacteriological analysis

An overview of the obtained results is shown in Table 6.3.

6.4.3.1. *Enterobacteriaceae/ Escherichia coli enumeration.*

The presence of Enterobacteriaceae varied per sample type: 3 out of 60 (5.0 %) raspberry/cherry tomato/strawberry samples were positive with a mean load ranging between 1.51 logs and 2.38 logs while 10 out of 15 (66.7 %) mixed fruit salad samples were positive with a mean load of 2.88 logs. *Escherichia coli* was not detected in any of the samples.

6.4.3.2. E. coli 0157, Salmonella and Listeria spp/monocytogenes.

Neither *E.coli* 0157 nor *Salmonella* could be shown in any of the tested samples, while 1 raspberry sample and 3 mixed fruit salad samples tested positive for *Listeria* spp.

Table 6.3 Overview bacteriological analysis of tested fruit samples.

Fruit type (25 g)	N	Enterobacteriaceae # positive samples (mean load [a])	*E. coli* # positive samples (mean load [a])	*Salmonella* [b]	*E.coli 0157:H7* [b]	*Listeria* spp*/monocytogenes* [b] # positive samples (specification)
Raspberries	10	2 (2.38)	0 (<1)	nd	nd	1 (L. *spp*)
Cherry tomatoes	30	1 (1.51)	0 (<1)	nd	nd	nd
Strawberries	20	0 (<1)	0 (<1)	nd	nd	nd
Mixed fruit salad	15	10 (2.88)	0 (<1)	nd	nd	3 (L. *spp*)
Total	75	13 (2.70)	0 (<1)	nd	nd	4 (L. *spp*)

[a] Values are expressed in log scale; [b] nd: not detected

6.5. Discussion

Various non-bacterial acute gastroenteritis outbreaks have been caused by intake of virally contaminated raspberries (Cotterelle et al., 2009; Falkenhorst et al., 2005; Le Guyader et al., 2004a; Ponka et al., 1999), strawberries (Hutin et al., 1999; Rutjes et al., 2006a), tomatoes (Zomer et al., 2009) and mixed fruit salads (Sivapalasingam et al., 2004). In addition, a number of bacterial food-borne outbreaks have been linked to consumption of similar fruit and vegetable products (Beuchat, 1996; Doyle and Erickson, 2008; Hanning et al., 2009).

In spite of these outbreaks linked to consumption of contaminated fruit products, only a number of authors have investigated the microbiological quality of these fruit products. In particular, the presence of enteric viruses on these food products has not been thoroughly investigated due to the lack of sensitive detection methods. Therefore, the present study was undertaken after the development and evaluation of a NoV detection methodology able to

detect 10^4 genomic copies per 10g of fruit product described in chapter 5. The murine norovirus-1 (MNV-1), a cultivable NoV surrogate was integrated in this detection methodology as full process control (MNV-1 PC), reverse transcription control (MNV-1 RTC) and real-time PCR internal amplification control (MNV-1 IAC).

The inclusion of MNV-1 in the NoV detection strategy was partially successful. While the MNV-1 RTC and IAC could successfully be recovered in 74/75 of all samples, the MNV-1 PC could only successfully be recovered in 31/75 of all samples. Quantitative analysis of the successful MNV-1 PC recoveries showed variable recovery efficiencies. It is therefore recommended that a higher concentrated MNV-1 PC ($\geq 10^5$ genomic copies per 10 grams of food product) should be used in further studies and when routinely screening fruit products for NoV in order to correctly interpret the recovery of this MNV-1 PC. Since it was expected that a higher concentrated MNV-1 PC would have resulted in a higher recovery success rate of this PC, NoV presence data were interpreted in two ways. On one hand (interpretation 1), a non-successful recovery of the MNV-1 PC was interpreted as inhibition of the sample and therefore no conclusions could be drawn from the sample, although a NoV positive sample was considered true positive, even with a non-successful recovery of the MNV-1 PC. Following this interpretation, no conclusions could be drawn from 36 out of 75 samples due to inhibition, while 39 out of 75 samples showed either a successful recovery of the MNV-1 PC or tested positive for NoV. On the other hand (interpretation 2), a non-successful recovery of the MNV-1 PC was interpreted as being caused by the low concentration of the MNV-1 PC and RT-PCR inhibition would be examined by analyzing the MNV-1 RTC and IAC. Following this interpretation, no conclusions could be drawn from a single (raspberry) sample.

The current study showed an unexpected high presence of NoV detected by real-time RT-PCR on the tested fruit samples. In total, 18 out of 39 (interpretation 1) or 74 (interpretation 2) tested fruit samples tested positive for GI and/or GII NoV, with concentrations between 2.5 to 5.0 logs per 10 gram of food product. It should be noted that maximally 2 out of 4 performed real-time PCR reactions per sample gave a positive signal in samples where NoV genomic presence was detected, which can be explained by the fact that most detected NoV concentrations were close to the presumed detection limit of the methodology. Since the observed Ct values of the positive samples ranged between 37 and 42, it is important to mention that all negative template controls (NTCs) were negative, thus excluding positive real-time PCR signals due to PCR contamination. Contamination-preventing measures such as the use of dedicated environmental conditions (separate working areas, UV and hypochlorite decontamination, dedicated pipettes) and the use of uracil DNA-glycosilase (UNG) containing real-time PCR mastermixes were respected at all time.

Due to the high number of real-time PCR positive results, confirmation of the results was attempted by subjecting NoV positive cDNA preparations, RNA preparations and virus extracts to three conventional RT-PCR assays used for genotyping NoV. Primer sets described by (Kojima et al., 2002; Vennema et al., 2002; Vinje et al., 2004) targeting respectively genotyping regions A, C and D were applied. However, no NoV sequences could be obtained despite intense efforts.

In a majority of the NoV positive samples, cloning of a (weak to very weak) PCR band of correct height failed, most likely due to insufficient material. The successfully cloned PCR bands from 2 cherry tomato samples and from the fruit salad sample could not confirm NoV presence by sequencing (data not shown). Most likely, the degenerated primer sets allowed aspecific amplification of genomic material of the food matrix.

It has been shown that real-time RT-PCR is 10^2 up to 10^4 fold more sensitive compared to conventional RT-PCR (Beuret, 2004; Pang et al., 2004), which may explain the failed confirmation since only 1 to 20 NoV genomic copies were detected by real-time RT-PCR in the cDNA of most positive samples.

Confirmation by obtaining sequences of fruits and vegetables samples that tested positive by real-time PCR has been tried by several research groups, but most of them were unsuccessful (Anderson et al., 2001; Makary et al., 2009; Wadl et al., 2010). A recent study investigating a cluster of NoV food borne outbreaks was able to confirm NoV presence in raspberries by conventional RT-PCR and subsequent sequencing (Maunula and von Bonsdorff, 2005).

Only a limited number of authors have investigated presence of enteric viruses on fruit products not related to food borne outbreaks. Recently, Mattison et al (2010b) found NoV presence in 148 out of 275 tested packaged leafy greens. Similar to our results, confirmation was also difficult although sequencing confirmed NoV presence in 16 out of 148 positive samples. A similar study examining the presence of GI and GII NoV, adenoviruses (AdV), enteroviruses (EV) and rotaviruses (RV) in irrigated vegetables was performed by (Cheong et al., 2009b). However, only 2 samples (lettuce and chicory) out of 30 tested positive for AdV, while a single spinach sample contained AdV as well as NoV.

It should be noted that the real-time PCR assay performed in the current study used primers and hydrolysis probes recommended by the CEN/TC275/WG6/TAG4 working group, which does not suggest confirmation as use of the hydrolysis probes should ensure the specific detection of NoV genomic material.

Ideally, only infectious NoV virus particles should be detected and the use of propidium monoazide in combination with real-time RT-PCR has been suggested for this purpose. Although this has been successfully tested for heat inactivated poliovirus, heat inactivated NoV was still detectable using this approach (Parshionikar et al., 2010). Another approach

for the specific amplification of infectious NoV is the treatment of virus extracts with RNase, but varying results have been reported. While RNase treatment has been shown to prevent RT-PCR amplification of heat inactivated feline calicivirus, poliovirus and hepatitis A virus, MNV-1 and human infective NoV could still be detected after a heat treatment and a hand sanitizer treatment (Baert et al., 2008b; Liu et al., 2009; Nuanualsuwan and Cliver, 2002).

Regarding the bacteriological quality of the tested fruit samples, analysis for *Enterobacteriaceae* on mixed fruit salad samples has been performed by Abadias et al. (2008) and a presence of 3.0 logs been noticed, which is similar to the presented results. *E. coli* 0157:H7 and *Salmonella* presence on various fruit products has been investigated by several authors, and similar to the current study, no presence of this pathogen has been reported (Bohaychuk et al., 2006; Johannessen et al., 2002). In contrast to a single study wherein the presence of *L. monocytogenes* has been reported on a strawberry sample (Johannessen et al., 2002), our results were in concordance with by Abadias et al. (2008) showing absence of this pathogen.

Despite the good bacteriological quality, an unexpected high presence of NoV was observed by real-time RT-PCR in particular in raspberries, strawberries and cherry tomatoes. However, it should clearly be noted that these positive real-time PCR results do not provide direct evidence for the presence of infectious NoV particles on the contaminated food products, since (q)PCR can only detect genomic material and thus cannot distinguish infectious and non-infectious NoV particles. For MNV-1, a 1 : 10^2 ratio of infectious virus particles : genomic copies has been noticed before on an untreated MNV-1 lysate solution, while such a 1 : 10^8 ratio was noticed when submitting this MNV-1 lysate to a heat treatment (Baert et al., 2008b). Therefore, development of methods able to discriminate infectious and non-infectious NoV particles in foods could clarify this matter.

6.6. Conclusions

In conclusion, results obtained in this chapter show the difficulty of expressing positive (real-time) PCR results towards terms of public health threat if no associated diseases or outbreaks are reported. Although these low NoV levels might indicate virus contamination at some point during the fresh produce chain, care should be taken to translate these results as a significant risk to the public health. Nevertheless, a possible risk for food borne transmission of NoV from these food products cannot be excluded either.

6.7. Acknowledgements

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General discussion and conclusions

7. CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The importance of noroviruses (NoV) as human pathogens present in the food chain has increased throughout the last years, as demonstrated by data from official agencies such as the European Food Safety Authority (EFSA) and Centers for Disease Control and Prevention (CDC) and the same vision is shared by the World Health Organization (WHO). This phenomenon can most likely be explained by the recent availability of molecular methods for detection of NoV, probably combined with an actual increase of the number of NoV food borne outbreaks.

Although a zoonotic hypothesis is under investigation (Bank-Wolf et al., 2010; Mattison et al., 2007), humans are considered the only reservoir for human infective NoV belonging to genogroups I (GI) and II (GII). Food products are in most cases contaminated with human infective NoV from this reservoir through two main transmission routes. A first main route of contamination of food with NoV is via contact with water contaminated with human faecal material and is mainly related to shellfish (by bio-accumulation of virus particles from water in harvesting areas) and produce (by use of contaminated irrigation water). A second main transmission route is through contact of the food products with an infected person such as the food handler or the food picker.

7.1. Development and evaluation of methods for detection of NoV in foods.

A broad range of foods has been involved in NoV food borne outbreaks. A review of 59 described NoV food borne outbreaks between 2000 and 2010 showed that mainly oysters, raspberries, deli sandwiches and composite meals were implicated in reported NoV food borne outbreaks (an overview is given in chapter 1 – Table 1.2).

However, laboratory confirmation of NoV presence from these foods was often hampered due to difficulties of detecting NoV in most foods. The main reason for this lies in the current inability to cultivate NoV (in contrast to most common food borne bacteria), despite intense efforts (Duizer et al., 2004). As a result, methods for detection of NoV in foods are generally complex and include in most cases 3 major steps: (1) extraction of the (RNA of the) virus from the food matrix, (2) purification of the extracted RNA and (3) molecular detection of the purified RNA.

A review of recent literature showed that several protocols have been described for each of these three steps (chapter 1 – paragraph 1.3). However, focus in most studies has been pointed towards development, optimization and comparison of NoV detection methods, while a thorough evaluation and validation of selected protocols has not been frequently

performed. Therefore, The **first goal** of this PhD consisted of the careful evaluation of current available NoV detection protocols specific for different food categories towards robustness and sensitivity. In an ideal case, an inter-lab evaluation should be performed, but this was considered to be out of the scope of this PhD. During such an inter-lab validation, a first phase could be the pointed towards comparison of the sensitivity of developed multiplex real-time RT-PCR (chapter 2) when used in different labs, and thus performed by different personnel/instruments using different real-time PCR systems. For this purpose, identical real-time PCR reagents (primers, hydrolysis probes, water, PCR mastermix) and plastic disposables could be distributed among different labs, as well as identical dilution series of a GI and GII NoV positive faeces sample (10^{-1} to 10^{-7} dilution in phosphate buffered solution (PBS)) and negative samples. For inter-lab evaluation of the use of MNV-1 as RTC and IAC, solutions with known concentrations of MNV-1 genomic RNA and plasmid p20.3 could be spread among the labs. A second step would then be focused on comparison of the sensitivity of the evaluated virus extraction protocols (chapters 4 and 5) between different labs. For this, soft red fruit samples (such as raspberries) or RTE foods (such as deli sandwiches) inoculated with a dilution series of a GI and GII NoV positive faeces sample would be diluted, accompanied with an MNV-1 solution of known concentration for evaluation of the use of MNV-1 as PC.

The first step in the construction of a defined protocol for thorough evaluation was the selection of primers and probes and the **development of a multiplex real-time reverse transciptase (RT-)PCR assay** (including an internal amplification control (IAC)) for detection of a broad range of human infective NoV and is described in chapter 2. While detection of NoV RNA can be performed by well-established techniques such as RT-PCR and NASBA with a likewise sensitivity in clinical samples (Houde et al., 2006), the evolution of these assays into a real-time format is somewhat easier for RT-PCR since (degenerated) hydrolysis probes can be used instead of molecular beacons. Many primer and hydrolysis probe sets for real-time RT-PCR detection of NoV have been described an compared (Vinje et al., 2004) and the European Committee for Standardization/ Technical Committee 275 / Working Group 6 /Task Group 4 on virus detection in foods (CEN/TC275/WG6/TAG4 working group) has proposed a set of primers and hydrolysis probes targeting the overlap between the first two open reading frames (ORF), considered to be the most conserved region in the dynamic NoV genome (Kageyama et al., 2003). Due to the genetic variability between the different genogroups, different primer – hydrolysis probe sets targeting this genomic region were proposed for GI and GII NoV. Noteworthy, a recent inter-lab comparison of (real-time) RT-PCR assays targeting this ORF1/ORF2 overlap confirmed that this region is still a valid target for detection of currently circulating GI and GII NoV genotypes (Mattison et al., 2010a). Primers and hydrolysis probes were thoroughly tested in chapter 2

and showed the ability to specifically detect genogroup I (GI) and II (GII) NoV. However, continued evaluation is needed to see whether this assay can detect new and emerging NoV genotypes such as the NoV GII.12 genotype and new NoV GII.4 variants. Equally important is the need to test developed primers and probes for the possibility to amplify aspecific sequences related to the tested food samples, to assure the specificity of results obtained when screening food products for NoV presence. Amplification of aspecific sequences related to the tested fruit matrices was considered unlikely during the NoV screening of 75 fruit samples in chapter 4, since very high Ct values were observed and a higher number of positive results would be expected (at lower Ct values). However, due to the inability to confirm the real-time PCR positive results obtained during this study, this aspecific cannot be fully ruled out. Therefore, further research on this matter could clarify whether aspecific amplification could be a problem when detecting NoV in foods.

To reduce the cost and hands-on time and in particular to take the option to include internal controls for reverse transcription and amplification, the CEN/TC275/WG6/TAG4 working group primers and hydrolysis probes for NoV GI and GII were combined with primers and a hydrolysis probe for detection of the murine norovirus 1 (MNV-1) into a multiplex real-time RT-PCR assay. Although the CEN/TC275/WG6/TAG4 working group suggested to use its primers in singleplex assays, both the individual singleplex assays and the multiplex assay developed in this PhD were capable of reliably detecting 10 NoV genomic copies per reaction, which is similar to other assays (Jothikumar et al., 2005b; Wolf et al., 2007). Noteworthy, a recent study comparing different real-time RT-PCR assays for detection of GI and GII NoV found that the use of a multiplex assay was significantly associated with false-negative results when detecting NoV in diluted faecal samples, in particular for the GI.2 NoV genotype (Mattison et al., 2010a). However, none of the laboratories used the multiplex setup (combination of primers and probes) as presented in chapter 2 and results using this setup showed that a broad range of GI and GII NoV could be detected, including several NoV GI.2 clinical/run-off RNA samples. Competition between the different reactions within the multiplex assay was investigated and found to be very limited, but the use of commercial real-time PCR mastermixes specifically developed for multiplex PCR may provide an added value towards the multiplex assay. While the CEN/TC275/WG6/TAG4 working group protocol suggested the use of reverse transcription and amplification controls in an external format, in the present PhD study it was preferred to use internal controls (in the same tube used for detection of GI and GII NoV) as this setup allowed a more reliable control of the real-time PCR reaction and reverse transcription, and also cover sample to sample and run to run variation in this manner.

Although the designed multiplex real-time RT-PCR assay has provided reliable results, an improvement could be realized by modification of this assay towards a “Linear After The

Exponential" PCR (LATE-PCR) assay. LATE-PCR, a specialized asymmetric PCR technique, makes use of different concentration for both primers (an 40 to 100 fold excess of one primer concentration) which avoids outcompetition of formation of primer:template strand hybrids because of the hybridization of synthesized template DNA strands (Pierce and Wangh, 2007). The length and composition of both primers is adjusted to compensate for this concentration difference (Pierce et al., 2005). This approach allows increased amplification efficiencies and higher fluorescence. A reported disadvantage is the fact that high concentrations of hydrolysis probe are needed (related to the concentration of the excess primer)(Sanchez et al., 2004). On the other hand, this technique could be combined with pyrosequencing, a sequencing technique based on the detection of the activity of the DNA polymerase by a chemiluminescent enzyme (Nyrén, 2007). The latter could be useful for confirmation of the specificity of the real-time PCR amplicon when detecting low levels of NoV in foods. Regarding the genotyping of NoV, a recently developed oligonucleotide array "NoroChip" has been designed for hybridization with a 917 bp RT-PCR product that encompassed genotyping regions B and C. Although the NoroChip allowed typing of all characterized NoV genotypes, the assay has only been evaluated on clinical samples. Further research is needed towards its use for genotyping of NoV in food samples (Pagotto et al., 2008).

For the reverse transcription step, a two-step protocol with the use of random hexamers was preferred over a one-step protocol. Although increasing the hands-on time, the two-step protocol was preferred based on the ability to use the prepared cDNA for other purposes such as genotyping or confirmation of positive samples.

Furthermore, during optimization of the NoV detection method in foods and selection of the appropriate **RNA purification method**, the automated NucliSens EasyMAG system (BioMérieux) provided better results for composite meals compared to the manual RNeasy minikit (Qiagen). In most cases however, both systems yielded comparable results. Both systems (or similar variant systems of the same manufacturer) have been compared for detection of NoV in soft red fruits (Butot et al., 2007), shellfish (Comelli et al., 2008) and clinical samples (Witlox et al., 2008). While the NucliSens EasyMAG provided better results in shellfish, both systems performed equally in clinical samples and in soft red fruits.

Following the development and evaluation of a sensitive real-time RT-PCR assay for detection of GI and GII NoV and MNV-1 (chapter 2), **protocols for extraction of the (RNA of) NoV from the food matrix** were thoroughly evaluated (chapter 4 and 5). As previously observed by Baert et al. (2008a), a single method for extraction of NoV from all food matrices is most likely not possible and three different food categories could be considered according to the components within the food matrix. A first category of foods contains mostly

carbohydrates and water, while proteins and fats are low abundant. This category consists mainly of fresh produce, of which raspberries and salad vegetables have been most frequently inflicted with NoV food borne outbreaks. A second category of foods contains fats and proteins rather than carbohydrates and covers a broad range of food products. In this category, composite meals and deli sandwiches have been mostly implicated with NoV food borne outbreaks. Bivalve molluscan shellfish were considered a third class, because of their ability to accumulate viral pathogens in the digestive tissue which allows development of virus detection methods specific for this food category. Examples of this food category often involved in NoV food borne outbreaks are oysters and mussels.

A proposal for standardized methods for extraction of NoV and other viral agents from fresh produce and bivalve shellfish has been launched by the CEN/TC275/WG6/TAG4 working group. On the other hand, a review of recent literature (chapter 1) showed that several methods for extraction of NoV (and other enteric viruses) exist for the category of foods composed of fats and proteins, but a generally accepted method is not available yet. The CEN/TC275/WG6/TAG4 working group did also not include this food category in their proposed methods. Since a wide variety of evaluated methods is available for virus detection in bivalve shellfish and NoV can reliably be detected in shellfish samples, focus in this PhD was not pointed towards this food category.

For soft red fruits (selected as typical carbohydrate/water based food), an alkaline elution – PEG precipitation approach described by Baert et al. (2008a), which is very similar to the protocol described by the CEN/TC275/WG6/TAG4 working group, was thoroughly evaluated (chapter 4). For the fat and protein based food category, a careful evaluation was performed (in chapter 3) of a promising direct RNA extraction protocol consisting of a long (TriConc) and short (TriShort) variant (Baert et al., 2008a). Noteworthy, the use of swabs is recommended by the CEN/TC275/WG6/TAG4 working group for hard surface carbohydrate foods such as tomatoes. Although the use of swabs would certainly reduce the presence of inhibitory substances in the virus extract, a comparison between both methods could determine which approach could yield better results. Nevertheless, the evaluated elution – precipitation approach was successfully used on cherry tomatoes during the NoV screening study on soft fruits in the present PhD study (chapter 6).Implementation and evaluation of these two types of extraction methods, the alkaline elution – PEG precipitation method for carbohydrate/water based foods and the direct RNA extraction protocol for protein/fat based foods, combined with the developed multiplex RT-PCR on a variety of food types within these two food categories, showed that the sensitivity of the NoV detection method is to a certain extent determined by dilution factors due to fractional use of prepared virus extract, purified RNA extracts or copy DNA (cDNA) preparations as shown in Fig. 7.1 . Consequently, the theoretical detection limits of the evaluated NoV detection methods were 40 (TriConc

protocol), 3200 (TriShort protocol) and 400 (elution-precipitation) NoV genomic copies per 10 g of food sample assuming the ability of the used real-time RT-PCR assay to detect 1 genomic copy per reaction. Reduction of these dilution factors could be obtained by modification of the RNA purification step and molecular detection. An increase of the virus extract volume used for RNA purification could be achieved by use of kits specified for RNA purification of larger volumes. This approach could be particularly useful for detection of low concentrations of NoV when using the elution-concentration protocol for soft red fruits or the TriShort variant of the direct RNA extraction protocol, as in these protocols only 1% to 10% of the virus extract is used for RNA extraction. An increase of the purified RNA volume used for reverse transcription or the use of one step real-time RT-PCR might also enable to reduce these dilution factors. However, the potential to increase sensitivity should also be evaluated towards the probable concentration of inhibitory substances.

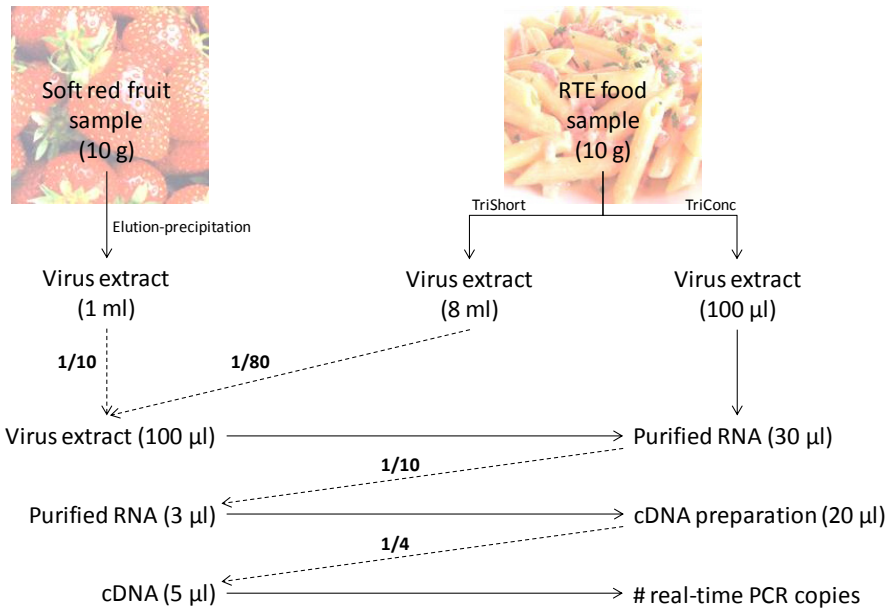

Fig. 7.1 Overview of dilution factors involved in the virus detection protocols from carbohydrate/water based foods and fat and protein based foods.

During carrying out of the experiments, the observed detection limit was 2- to 100-fold higher compared to the theoretical detection limit due to the recovery efficiency. Throughout the evaluation of the established NoV detection methods, a clear influence of the NoV inoculum level on its recovery was noticeable as high inoculum levels were recovered more successfully and with a higher efficiency compared to low level inocula. This phenomenon

was more explicit for the direct RNA extraction protocol. The influence of the food type on the recovery was more explicit on the elution-precipitation protocol compared to the direct RNA extraction protocol. Especially recovery of low level NoV (~10^4 genomic copies / 10g) from deli sandwiches was significantly more difficult compared to other foods such as soups or composite meals. To reliably detect NoV from this food matrix, the direct RNA extraction protocol could be modified to prevent the absorption of the Tri-Reagent® by the bread matrix after the centrifugation step which might be the cause of this difficulty. A second approach to detect NoV and other enteric viruses from deli sandwiches is by swabbing the outsides of the sandwich instead of using entire food parts. This approach could be supported by the fact that a food handler was involved in most food borne NoV outbreaks related to consumption of deli sandwiches, making it more likely that the outside of the deli sandwich is contaminated.

Overall, the alkaline elution – PEG precipitation protocol was able to recover NoV from soft red fruits with efficiencies of 10 % to 30 % in most cases while the direct RNA extraction protocol yielded recovery efficiencies of >1% (TriShort protocol) and 0.1 to 10 % (TriConc protocol). For both NoV extraction methods, taking into account all dilution factors resulted in a detection limit of approximately 10^4 genomic copies/10g. Simultaneous recovery of GI and GII NoV in similar or 100-fold different concentrations was possible in both food categories. It should be noted however, that all evaluation studies were performed on inoculated samples and further evaluation of the NoV detection methodology on naturally contaminated samples is thus needed. The direct RNA extraction method (chapter 4) has successfully been used in the National Laboratory for Foodborne Outbreaks in Belgium in 2007 for detection of NoV in deli sandwiches and composite meals related to NoV food borne outbreaks (Baert et al., 2009b), although a different real-time PCR assay was applied (Jothikumar et al., 2005b). Currently, the primers and hydrolysis probes for GI NoV, GII NoV and MNV-1 evaluated in chapter 2 are used for real-time PCR detection of NoV in this lab.

7.2. Evaluation of the use of MNV-1 as a control reagent for NoV detection in foods.

NoV levels in foods (whether or not related to food borne outbreaks) were expected to be close to the detection limit of the presented NoV detection methods and food matrices are known to contain substances able to inhibit enzymatic activity. Therefore, the **second goal** of this PhD consisted of the evaluation of the **murine norovirus 1** (MNV-1), a cultivable genogroups V NoV, as **control reagent**.

MNV-1 was used to control the entire virus detection protocol (process control; PC), the reverse transcription reaction (reverse transcription control; RTC) and the real-time PCR reaction (internal amplification control; IAC) when detecting NoV in foods. The use of these controls was combined to obtain more specific information on which steps of the NoV

detection methods are more susceptible to inhibition and which steps might reduce the overall sensitivity. The inclusion of a process control and an internal amplification control has been suggested for detection of foodborne viruses by the Belgian Superior Health Council (2010), while ISO 22174:2005 required the use of an adequate PC and IAC for detection of food borne pathogens by PCR.

The use of MNV-1 as PC was chosen as control reagent besides other successfully tested viruses because of the closer genomic relationship to human infective NoV. Other successfully used PCs for detection of viral pathogens in food include the feline calicivirus (FCV), F-RNA bacteriophage MS2, and a genetically modified mengovirus (vMC_0). vMC_0 has been proposed as process control for detection of HAV in shellfish samples (Costafreda et al., 2006), but its use is restricted in some laboratories because of the genetic modification of this virus. The feline calicivirus (FCV) on the other hand, can be considered as an adequate process control when detecting NoV in foods, but questions have been raised towards the suitability of FCV as NoV surrogate in inactivation studies due to an increased sensitivity towards acid environments. Additionally, while all mentioned viruses have similar size as human infective NoV (27 – 32 nm diameter), the isoelectric point of FCV and the MS2 bacteriophage is lower, while the exact isoelectric point of MNV-1 and vMC_0 is not known yet (Michen and Graule, 2010). This isoelectric point has an influence on the binding of the virus to food surfaces (Vasickova et al., 2010; Vega et al., 2005), and further research might therefore point out if one of these viruses binds more similarly to the food matrix and is thus more suitable as process control. Currently, the CEN/TC275/WG6/TAG4 working group suggests vMC_0 as process control and CEERAM (Centre Européen d'Expertise et de Recherche sur les Agents Microbiens; La Chapelle sur Erdre Cedex, France) has developed the "mengo@ceeramTools™" kit to control virus detection on food and clinical samples using vMC_0 (www.ceeramtools.com/pdf/ceeram-tools.pdf).

The use of MNV-1 as PC was successful in several soft red fruits and RTE foods, but a sufficient high concentration (10^6 genomic copies/10 g food product) is needed to assure detection and reliable estimation of its recovery efficiency. If used in lower concentrations, detection of the MNV-1 PC may not be possible in some food types as shown during the NoV screening of fruit products in chapter 6. To avoid interference with the multiplex real-time RT-PCR detection of NoV GI and GII, MNV-1 was used as an external process control (in parallel with a sample tested for GI and GII NoV). Both in carbohydrate/water based foods and fat and protein based foods, recovery efficiency of the MNV-1 PC was in most cases similar to recovery of the GI/GII NoV, if inoculated in same levels (10^6 genomic copies/10g). It should be noted that the use of MNV-1 as PC when detecting NoV in shellfish using a proteinase K treatment has yielded very low recoveries (De Naeyer et al., in preparation) and further research on this matrix could therefore clarify this matter.

The use of MNV-1 to control the reverse transcription reaction and real-time PCR assay was considered necessary because both reactions are prone to inhibition, in particular when analyzing foods (Loisy et al., 2005; Love et al., 2008). The reverse transcription reaction and real-time PCR were controlled by addition of MNV-1 RNA and a plasmid containing a full cDNA copy of the MNV-1 genome to the reaction mixes, respectively. Concentrations of MNV-1 RTC and MNV-1 IAC used were respectively 10^4 RNA copies/reaction and 10^2 plasmid copies/reaction to avoid influence of the outcome of the real-time PCR assay, especially in case of low GI/GII NoV concentrations. Although the CEN/TC275/WG6/TAG4 working group proposed the use of external reverse transcription controls and external amplification controls, it is our belief that the use of these controls in an internal format has several advantages. While sample-to-sample variations are detectable by internal controls, this setup also allows a reduction of the number of performed PCR reactions.

Limited recovery (<20 %) of the MNV-1 RTC was only occasionally noticed in carbohydrate/water based foods which suggests that in most cases the virus extraction protocol sufficiently removed inhibitory substances. The use of MNV-1 RTC was not thoroughly evaluated in the direct RNA extraction protocol (chapter 4) as the focus was rather placed on the robustness of the method by subjecting a broad variety of inoculated food materials to the direct RNA extraction protocol. However, the RT reaction can be inhibited when testing on meat and the failure to recover the low level NoV inocula from several RTE foods might thus be related to this problem (McIngvale et al., 2002). Similar to the reverse transcription, inhibition of real-time PCR assay was only rarely observed, both in carbohydrate/water based and fat and protein based foods although recovery efficiency of the MNV-1 IAC was generally higher compared to the recovery of the MNV-1 RTC. Plant material is known for inhibition of PCR (Tzanetakis et al., 2005), which suggests that the elution-precipitation succeeded in most cases to remove inhibitory substances.

In conclusion, results obtained during evaluation of MNV-1 as RTC and IAC suggest that the reverse transcription reaction is more prone to inhibition compared to the real-time PCR reaction when working in foods. In particular for the reverse transcription, the use of commercial mastermixes or components that offer protection against this inhibition could aid in reducing this difficulty.

From evaluation of the use of MNV-1 as PC, RTC and IAC, a shortened NoV detection strategy for soft red fruits and RTE foods could be suggested (Fig 7.2). In this strategy, the use of MNV-1 IAC is left out, which reduces the number of PCR reactions testing for GI and GII NoV from 2 to 1 compared to the NoV detection strategy proposed in chapter 5 (Fig 5.1), which could lead to possible false-negative results as shown by the screening of fruit products in chapter 6. This could be resolved by testing the subsample inoculated with the MNV-1 PC by multiplex real-time RT-PCR for GI and GII NoV as well. To avoid competition

between the individual reactions within the multiplex assay, the concentration of the MNV-1 PC should be limited to 10^6 genomic copies / 10 g food.

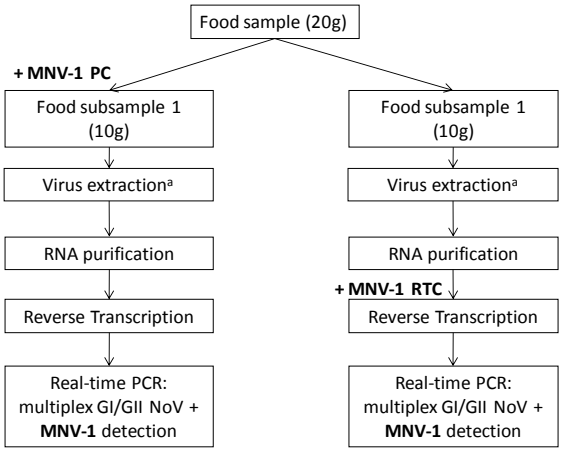

Fig. 7.2. Proposed detection strategy including MNV-1 as process control (MNV-1 PC) and reverse transcription control (MNV-1 RTC). [a] Virus extraction: the evaluated direct RNA extraction method (TriShort or TriConc variant; chapter 4) for RTE foods or the evaluated elution-precipitation protocol (chapter 5) for soft red fruits.

7.3. The difficulty of correctly interpreting PCR results.

Following the NoV prevalence study (chapter 6) using the NoV detection method described in chapters 2 and 5, it became clear that **correct interpretation of real-time PCR results** is not always straightforward and can be influenced by several factors, especially when detecting low levels of NoV in foods. This can make translation of positive PCR results to a decision on whether or not the NoV positive samples (PCR positive) pose a significant risk to public health (and thus warrant a recall or any other action) difficult. While in case of a food borne outbreak, interpretation of food products as a threat to public health due to positive PCR results could be supported by epidemiological data showing related illness, this not the case for preventive NoV screening of food products such as fresh produce. Considering these difficulties, several criteria should be analyzed for correct interpretation of these PCR results.

First of all, the results of **controls** that are taken up in the test protocol should be examined. Positive controls (either MNV-1 PC, RTC or IAC) are necessary to trace inefficient virus extraction (process control) or inhibited enzymatic activity (RTC and/or IAC). A problem often encountered but not frequently reported is the presence of PCR contamination leading to positive no template controls (NTCs) and thus to potentially false-positive results. During development of the multiplex real-time RT-PCR assay, positive NTCs were frequently observed and extensive testing suggested migration of the positive PCR controls (synthetic

100 nucleotide ssDNA fragments) between wells, leading to the positive NTCs. Cloning of the short DNA fragments into commercial vectors and using these plasmids as positive controls has resolved the contamination problem and no consistent contamination problems have been observed further.

In general however, the increased sensitivity of molecular methods might lead to an increase of false-positive results because of contamination, especially in routine laboratories. Therefore, several measures can be taken to avoid this problem when using RT-PCR in a laboratory. A first preventive measure consists of the use of physically separated working places with dedicated pipettes, gloves and lab coats for the different steps of the preparation of the reverse transcription reaction and PCR. A first working place should be used for preparation of the RT-PCR mastermixes (pre-RT-PCR work) and only RT-PCR reagents (primers, probes, water, DNA polymerase, reverse transcriptase and other RT and PCR components) should be allowed in this working place, while template DNA and RNA must not be held storage or manipulated in this place. All RT-PCR reagents should be divided in aliquots and stored in an area free of target RNA and DNA. If possible, the pre-RT-PCR working place should be held at an overpressure to avoid template RNA and DNA from entering. A second working place should be separated for addition of the template DNA and RNA to the PCR and reverse transcription reaction, respectively. If possible, this place should be held at underpressure to avoid template DNA and RNA from leaving. A third working place should be used for detection (and confirmation) of RT-PCR products. In most labs, a separated room for confirmation is required as well, because of the use of toxic chemicals such as ethidium bromide. Finally, sample preparation (for extraction of RNA or DNA from pathogens) should be performed in separate working places specified for this work (in particular for RNA work). A second preventive measure consists of the use of PCR kits with Uracil N-glycosylase and deoxyuridine triphosphate (dUTP) rather than deoxythymidine triphosphates (dTTP), a system designed for prevention of carryover-contamination from previous PCRs (Kleiboeker, 2005; Longo et al., 1990; Pang et al., 1992). A final preventive measure consists of the use of shortwave UV irradiation and hypochlorite solutions (3% m/m) to remove contaminating nucleic acids from working space. However, it is almost impossible to avoid occasional contamination, especially in routine labs. Therefore, a constant alertness is required for problems concerning RT-PCR contamination even if these measures are respected.

Secondly, **experienced scientific personnel** is required to correctly analyze real-time PCR data. Although in theory the sigmoidal shape distinguishes positive signals from background signals, this is not always obvious when detecting low levels of NoV in foods. Moreover, NoV RT-PCR positive results from the NoV screening study were obtained at higher Ct values (Ct

37 to 42) and were often not reproducible (1 or 2 out of 4 PCRs positive) indicating that the method was pushed or being used close to the detection limit.

A third issue for correct interpretation of positive PCR results is their **confirmation** by amplification of a different and larger genomic region. Although this could provide (1) assurance of the specific detection of NoV genomic material and (2) more information of the detected NoV genotype in the food sample, confirmation is often very difficult when detecting low levels of NoV in foods. This problem was encountered during the NoV prevalence study in soft red fruits (chapter 6) as none of the positive real-time PCR results could be confirmed by conventional RT-PCR assays targeting genotyping regions A, C and D (Mattison et al., 2009b) despite intense efforts. A possible explanation might be that the conventional RT-PCR assays target a larger genomic fragment compared to the real-time RT-PCR assay, which might result in a reduced amplification efficiency. Noteworthy, the CEN/TC275/WG6/TAG4 working group stated that confirmation of the real-time PCR results is deemed unnecessary since the use of hydrolysis probes assures the specific detection of NoV genomic material. In bacteriology, confirmation by cultivation is often required in case a positive PCR is encountered. However, detection of NoV is currently not possible due to the inability for cultivation, in contrast to most common foodborne pathogens. Therefore, only PCR results (often close to detection limit) can be consulted for analysis of NoV presence on a food sample.

7.4. Interpretation of PCR results towards public health safety.

Even when a PCR result is regarded as correctly positive (whether or not with confirmation), the answer to the question if food products that test positive for NoV by PCR could form a threat to public health is still not straightforward.

First of all, a major concern of the use of molecular techniques for the detection of NoV in food is their incapability to distinguish **infectious and non-infectious NoV particles**, since only genomic material is detected (Nuanualsuwan and Cliver, 2002). This important disadvantage should be taken into account if PCR results should be used to declare if food matrices are safe for consumption, as no data is currently available for the influence of factors such as pH, temperature and time on the infectivity of human infective NoV. Development of methods able to distinguish infectious and non-infectious NoV particles would allow to detect and quantify the number of infectious virus particles in the tested food products. Recent efforts have been made on this field through integrated cell culture RT-PCR (ICC RT-PCR), a technique combining cell culture and RT PCR. Its principle is based on the assumption that a discrimination can be made between infectious and non-infectious virus particles by infecting specific cell lines (Chapron et al., 2000; Reynolds et al., 1996). Although NoV cannot be cultivated despite testing on several cell lines (Duizer et al., 2004),

NoV particles have been shown to bind to CaCo-2 cells and pig stomach mucin (Tian et al., 2005; White et al., 1996). Using this binding properties, efforts are currently going on to develop an ICC RT-PCR able to discriminate infectious and non-infectious NoV particles (Li et al., personal communication). Another effort has been made through the use of propidium monoazide (PMA), a DNA intercalating dye with a photo-inducible azide group that covalently cross-links to DNA upon exposure to bright light (Nocker and Camper, 2009). Discrimination of infectious and non-infectious virus particles by PMA is based on two principles. PMA cannot migrate through infectious virus capsids (Parshionikar et al., 2010) and binding of PMA to DNA or RNA makes these molecules unavailable for PCR amplification. Therefore, treating samples with PMA could be applied for discrimination of infectious and non-infectious virus particles, as PMA would only bind to RNA of compromised capsids of non-infectious or inactivated viruses. In a similar way, PMA has been used to distinguish between viable and nonviable bacteria (Nocker et al., 2006; Pan and Breidt Jr, 2007), and recent research has shown that this approach could be used in some cases for discrimination of infectious and non-infectious NoV particles (Parshionikar et al., 2010). From a public health protection perspective, translation of these techniques towards food products contaminated with low levels of NoV could provide information towards the risk of exposure to NoV as well as to other food borne viruses.

Secondly, the increased sensitivity of methods for detection of NoV (and other enteric viruses) in foods may result in a further **increased detection** of these **viruses** in tested foods.

If a NoV outbreak has been related to a food product, detection of the presence of NoV in the food product by PCR may clarify the possible transmission route and may support eventual removal of the food product from the market. In particular, the presence of identical NoV genomic sequences in food product(s) and in clinical samples from a NoV foodborne outbreak may provide evidence. On the other hand, screening of NoV presence in food products not related to NoV outbreaks may also result in a further increased NoV detection due to this increased sensitivity. The NoV screening study described in chapter 6 showed an unexpected high number of produce samples of good bacteriological quality testing positive for NoV, in most cases with NoV concentrations close to detection limit. In detail, 18 out of 74 fruit samples tested positive with detected NoV genomic copy concentrations ranging between 2.5 and 5.0 logs per 10g of tested fruit products and with a maximum of 2 out of 4 PCR reactions testing positive. Recently, a similar more extensive screening of 1020 various food products not implicated in food borne outbreaks showed that 8.3 % and 18.5 % of these foods tested positive for NoV GI and GII, respectively. Of all food samples, 4.8 % contained NoV levels over 10^4 genomic copies / 25 g (Fabienne Loisy, personal communication / unpublished results). Currently, food products testing positive for NoV are either prevented

from reaching the market or withdrawn from it (Rapid alert system for Food and Feed (RASFF) portal, subject: norovirus; "https://webgate.ec.europa.eu/rasff-window/portal/"). While this "better-safe-than-sorry" approach is defendable towards public health, this can have serious economical implications for companies manufacturing, distributing and selling these food products. Because of this apparent widespread presence of (genomic material of) NoV, it is worth investigating if a NoV level threshold – whether or not linked to a real-time RT-PCR cut-off Ct value – could be applied to determine if foods are considered a threat to public health. This threshold could provide more information to support eventual removal of these food products from the market. Although a study has shown that NoV positive shellfish samples are not necessarily related to food borne outbreaks, regardless of the NoV levels (Lowther et al., 2010), further studies connecting the NoV load on foods to reported illness could be conducted to investigate this proposal.

7.5. Added value of this PhD

NoV have worldwide increasingly been recognized as important food borne pathogens. However, the limited number of laboratory confirmed food samples as cause of NoV outbreaks point towards a lack of sensitive methods for detection of NoV in food products. Therefore, a critical evaluation of recently developed methods for detection of NoV in foods towards sensitivity and robustness was considered necessary. A methodology enabling detection of NoV from soft red fruits and fat and ready-to-eat (RTE) foods was designed and evaluated. The murine norovirus 1 (MNV-1) was used as control reagent at different steps in these methodologies. A screening study of NoV on produce products present on the market was conducted upon successful evaluation of the NoV detection methodology. Following points were investigated and contributed to this research.

- *Development of a multiplex real-time RT-PCR assay for simultaneous detection of GI/GII NoV and MNV-1.* By combining specific primers and hydrolysis probes, real-time PCR detection of GI/GII NoV and MNV-1 was possible in a single reaction, wherein reliable detection of 10 genomic copies was possible. While this setup allowed the use of MNV-1 as real-time PCR IAC, it reduces both time and costs as well.
- *Real-time PCR contamination issues.* It was shown that the use of short single stranded DNA fragments as positive control can lead to false-positive real-time PCR results. The use of plasmids as positive controls resolved the problem of frequent occurrence of positive NTCs in the real-time PCR assay.
- *Virus extraction method and RNA purification.* The sensitivity and robustness of two methods, described by Baert and colleagues (2008a extraction of (genomic material) of NoV were tested. For soft red fruits and RTE foods, an elution-concentration

protocol and a direct RNA extraction protocol were respectively evaluated. It was demonstrated that both protocols allowed reliable detection of 10^6 genomic copies per 10 g of food product, while the detection limit of both methods was approximately 10^4 genomic copies of GI/GII NoV per 10g of food product.

- *NoV prevalence on soft fruits.* Screening of 75 fresh produce samples (raspberries, strawberries, cherry tomatoes and fruit salads) showed an unexpected high presence of GI and GII NoV on fresh produce, despite good bacteriological quality. Questions were raised whether or not these food products could form a threat to public health safety.

In conclusion, the development of a multiplex real-time RT-PCR assay for detection of human infective NoV combined with the evaluation of food category specific virus extraction methods and accompanying RNA purification methods have contributed to the acceptance of a standardized method for NoV detection in foods. Nevertheless, further research could improve the detection of NoV in food matrices such as deli sandwiches. The integration of these NoV detection methodologies in reference laboratories would allow the reliable investigation of (the increasing number of) NoV food borne outbreaks.

However, the use of sensitive NoV methods has revealed the difficulty of correctly interpreting positive PCR results towards public health safety. Therefore, an increased focus towards the development of guidelines for interpreting positive PCR results as well as the development of methods able to specifically detect infectious NoV could aid to this matter.

Summary - Samenvatting

SUMMARY

In this PhD, three main goals were defined. The first goal consisted of the development and evaluation of a methodology for detection of noroviruses (NoV) in ready-to-eat (RTE) foods and soft red fruits while the second main goal included the evaluation of the murine norovirus 1 (MNV-1) as control reagent for different steps throughout the NoV detection protocols. Finally, a screening study on a selection of fruit produce products towards NoV presence was the third main goal of this PhD.

To illustrate these goals, a literature study was performed in **chapter 1**. In this literature study, a brief overview of the most important food borne viruses was followed by a more detailed description of NoV in terms of classification, virion and genome structure. The NoV genotype most commonly identified in NoV gastroenteritis outbreaks (NoV GII.4) was described as well. The importance of NoV as a food borne pathogen was highlighted by data originating from official bodies such as CDC (Centers for Disease Control and Prevention; USA) and EFSA (European Food Safety Authority; Europe), accompanied with data gathered on own initiatives by research groups. The two main transmission routes of NoV contamination of foods (pre-harvest contamination via contact with contaminated water and (post-) harvest contamination via an infected food handler/picker) were investigated by summarizing and analyzing 59 NoV food borne outbreaks described between 2000 and 2010. Furthermore, the three main steps of NoV detection in food were portrayed in detail to prepare the development and evaluation of the NoV detection methodology in chapters 2, 3, 4 and 5. Finally, the use of adequate positive and negative controls to assure reliable detection of NoV in foods was illustrated. For the first and second goals of this PhD, a molecular assay for detection of the purified NoV genomic material was optimized and subsequently combined with protocols for extraction of (genomic material of) NoV from RTE foods and soft red fruits in chapters 4 and 5. MNV-1 was included in these protocols as control reagent.

The molecular detection assay described in **chapter 2** was a quantitative two-step multiplex real-time reverse transcriptase (RT-) PCR assay for simultaneous detection of NoV genogroup I (GI) and II (GII) and the murine norovirus 1 (MNV-1), the latter used as internal amplification control (IAC). For this multiplex assay, NoV GI and GII specific primers and hydrolysis probes designed by the European Committee for Standardization/ Technical Committee 275 / Working Group 6 /Task Group 4 on virus detection in foods (CEN/TC275/WG6/TAG4 working group) were combined with primers for murine norovirus 1 designed by Baert and colleagues (2008b). Evaluation of this multiplex assay showed a high concordance between the multiplex assay and the corresponding singleplex PCR assays.

Specificity analysis of the multiplex assay by testing a NoV RNA reference panel and clinical GI and GII NoV samples showed that specific amplification of NoV GI and GII was possible. In addition, no cross-amplification was observed when subjecting a collection of bovine NoV and other (non-NoV) enteric viruses to the multiplex assay. Finally, MNV-1 was successfully integrated as IAC, although a sufficiently low concentration was needed to avoid interference with the possibility of the developed multiplex assay to quantitatively and simultaneously detect the presence of GI and GII NoV within one sample.

During development of the multiplex real-time RT-PCR assay, contamination issues were encountered and the investigation towards the source of the positive no template controls (NTCs) was described in **chapter 3**. This investigation was believed to be necessary because of the need for reliable detection of 10 or less NoV genomic copies per PCR reaction, due to the low infectious dose of GI and GII NoV. In this chapter, a suspicion of well-to-well migration of positive control DNA (a short synthetic single stranded DNA (ssDNA) fragment) during real-time PCR runs was uttered as hypothetic cause of the positive NTCs. Results in this chapter showed that evaporation of water occurred during real-time PCR runs regardless of the DNA type, the reaction plate seal type and the use of mineral oil as cover layer. It was also suggested that co-evaporation of DNA took place, with an apparent negative correlation between the size of the DNA type and the extent of this co-evaporation. The use of mineral oil as cover layer and plasmid DNA as quantitative positive PCR control resulted in a complete absence of positive NTCs while only negligible effects were noticed on the performance of the real-time PCR.

After development of the multiplex real-time RT-PCR assay and the resolving of the contamination issues, two protocols for extraction of (genomic material) of NoV from foods were evaluated towards robustness and sensitivity while MNV-1 was evaluated as process control in both protocols.

The evaluation of a direct RNA extraction protocol for extraction of NoV genomic material (RNA) from RTE foods was described in **chapter 4**, while the evaluation of an elution-concentration protocol for extraction of NoV from soft red fruits was illustrated in **chapter 5**. For the RTE foods, the direct RNA extraction protocol made use of a guanidine isothiocyanate containing reagent to extract viral RNA from the food sample (basic protocol called TriShort), followed by an eventual concentration step using organic solvents (extended protocol called TriConc). The protocol for extraction of NoV from soft red fruits consisted of alkaline elution of NoV particles from the food, followed by polyethylene glycol (PEG) precipitation and organic solvent purification. For both protocols the RNA was subsequently purified. This purified RNA was detected by the multiplex real-time RT-PCR assay as described in chapter 2. To evaluate both NoV extraction methods towards sensitivity and

robustness, the influence of (1) the NoV inoculum level and (2) different food types on the recovery of NoV from these foods was investigated.

First of all, a significant influence of the NoV inoculum level on the recovery of NoV from foods was demonstrated for both protocols. High level inocula could be recovered from penne salad, selected as typical RTE food, with higher recovery success rates compared to low level inocula. For these high inoculum levels, the TriShort and TriConc protocols resulted in mean recovery efficiencies of >1 % and 0.1 to 10 %, respectively. Recovery of these low and high level NoV inocula from frozen raspberry crumb was possible with high recovery success rates and with mean recovery efficiencies of 10 to 30 % in most cases.

Secondly, a significant influence of the food type on the recovery of NoV could be shown for both protocols. For the direct RNA extraction protocol, the TriConc protocol provided better NoV recoveries for soups, while TriShort and TriConc protocols performed likewise for composite meals and deli sandwiches, although NoV recovery from the latter food type was problematic. For the elution-concentration protocol, a significant influence of the soft red fruit product type on the recovery efficiency of NoV GI and MNV-1 was noticeable, while no significant differences could be shown for GII NoV. In general, the recovery of NoV was more efficient and successful from the strawberry puree compared to a frozen forest fruit mix and fresh raspberries.

Regarding the evaluation of MNV-1 as control reagent, results from chapter 4 and chapter 5 suggested that a sufficient high concentration of the MNV-1 PC was needed to allow an estimation of possible inhibition of the RT-PCR or of inefficient virus extraction. When used as reverse transcription control or internal amplification control, the concentration should be adjusted to avoid interference with the quantitative properties of the developed multiplex real-time RT-PCR assay.

Chapter 6 described the screening of 75 fruit products (raspberries, strawberries, cherry tomatoes and fruit salads) for NoV presence using the virus extraction protocol described in chapter 5 combined with the multiplex real-time RT-PCR assay illustrated in chapter 2. In total, 18 samples tested positive for GI and/or GII NoV genomic material despite a good bacteriological quality. The level of detected NoV genomic copies concentrations ranged between 2.5 and 5.0 logs per 10 grams of fruit sample. NoV GI and/or GII were found in 4/10, 7/30, 6/20 and 1/15 of the tested raspberries, cherry tomatoes, strawberries and fruit salad samples, respectively. However, confirmation of the positive real-time PCR results by sequencing genotyping regions in the NoV genome was not possible. The question whether or not these unexpected high number of NoV positive results obtained should be perceived as a public health threat was raised and discussed.

In conclusion, methods for detection of NoV in RTE foods and soft red fruits were developed and evaluated towards sensitivity and robustness. For detection of NoV in soft red fruits and ready-to-eat foods, an elution-precipitation protocol and a direct RNA extraction protocol were combined with an optimized multiplex real-time RT-PCR assay leading to NoV detection protocols with detection limits of ~10^4 genomic copies / 10g food product. Influence of the NoV inoculum level and food type on NoV recovery was shown. Additionally, MNV-1 was successfully evaluated as control reagent, and suggestions were made towards its use. However, application of the method for NoV detection in fruit products has shown that interpretation of NoV presence by molecular methods is not straightforward and raises several questions, especially towards the public health safety.

SAMENVATTING

In dit doctoraatsonderzoek werden drie hoofdoelstellingen gedefinieerd. De eerste doelstelling bestond uit de ontwikkeling en evaluatie van een methodologie voor de detectie van Norovirussen (NoV) in kant-en-klare voedingsmiddelen en zacht rood fruit, terwijl de tweede hoofddoelstelling de evaluatie van het murine norovirus 1 (MNV-1) als controle reagens voor verschillende stappen doorheen het NoV detectie protocol omvatte. De derde en laatste doelstelling hield een screening in van een selectie aan fruitstalen voor de aanwezigheid van NoV.

Om deze drie doelstellingen te illustreren, werd een literatuurstudie uitgevoerd, beschreven in **hoofdstuk 1**. In deze literatuurstudie werd een kort overzicht van de meest belangrijke voedselgebonden virussen gevolgd door een meer gedetailleerde beschrijving van NoV omtrent classificatie, virion- en genoomstructuur. Het meest voorkomende NoV genotype in NoV gastro-enteritis uitbraken (NoV GII.4) werd eveneens beschreven.

Het belang van NoV als een voedselgebonden pathogeen werd geïllustreerd met behulp van data afkomstig van officiële instellingen zoals CDC (Centers for Disease Control and Prevention; VSA) en EFSA (European Food Safety Authority; Europa) en met behulp van data verzameld op eigen initiatief door onderzoeksgroepen.

De twee belangrijkste transmissieroutes voor NoV contaminatie van voedingsmiddelen (pre-harvest contaminatie veroorzaakt door contact met gecontamineerd water en (post-) harvest contaminatie veroorzaakt door een geïnfecteerde voedselbehandelaar/voedselplukker) werden toegelicht aan de hand van een overzicht van 59 voedselgebonden NoV uitbraken, beschreven tussen 2000 en 2010. Daarnaast werden de drie belangrijkste stappen in de NoV detectie in detail geïllustreerd ter voorbereiding van de ontwikkeling en evaluatie van de NoV detectie methodologieën in hoofdstukken 2, 3, 4 en 5. Tenslotte werd het belang weergegeven van geschikte positieve en negatieve controles om betrouwbare detectie van NoV in voedingsmiddelen te kunnen verzekeren. Voor de eerste en tweede doelstelling van dit doctoraatsonderzoek werd een moleculaire test voor de detectie van opgezuiverd NoV genomisch materiaal (geoptimaliseerd in hoofdstuk 2) gecombineerd met protocols voor de extractie van (genomisch materiaal van) NoV uit kant-en-klare levensmiddelen en zacht rood fruit (geëvalueerd in hoofdstukken 4 en 5). In deze protocols werd MNV-1 geïntegreerd als controle reagens.

De moleculaire detectie test beschreven in **hoofdstuk 2** omvatte een kwantitatieve twee-staps multiplex real-time reverse transcriptase (RT-) PCR voor simultane detectie van NoV GI, GII en het murine norovirus 1 (MNV-1), waarbij deze laatste gebruikt werd als interne

amplificatie controle (IAC). Voor deze multiplex PCR werden NoV GI en GII specifieke primers en hydrolyse probes, ontwikkeld door de “European Committee for Standardization/ Technical Committee 275 / Working Group 6 /Task Group 4 on virus detection in foods” werkgroep (CEN/TC275/WG6/TAG4 werkgroep), gecombineerd met primers voor MNV-1, ontwikkeld door Baert en collega’s (2008b). Evaluatie van deze test toonde een hoge overeenkomst aan tussen de multiplex PCR en de overeenkomstige singleplex PCR reacties. Analyse van de specificiteit van de multiplex PCR werd uitgevoerd door een NoV referentie panel en klinische NoV GI en GII stalen te onderwerpen aan de PCR. Resultaten toonden aan dat specifieke amplificatie van NoV GI en GII mogelijk was en bovendien werd geen cross-amplificatie waargenomen wanneer een collectie van dierlijke NoV en andere (niet-NoV) enterische virussen aan de multiplex PCR werd onderworpen. Tot slot werd MNV-1 succesvol geïntegreerd als IAC, mits gebruik van een gepaste concentratie om interferentie met de kwantitatieve eigenschappen van de multiplex PCR te vermijden.

Gedurende de ontwikkeling van de multiplex real-time RT-PCR werden contaminatieproblemen waargenomen en het onderzoek naar de oorzaak van de positieve no template controles (NTCs) werd beschreven in **hoofdstuk 3.** Onderzoek werd geacht noodzakelijk te zijn door de nood aan betrouwbare detectie van 10 of minder NoV genomische kopieën per PCR reactie, dit omwille van de lage infectieuze dosis van GI en GII NoV. In dit hoofdstuk werd het vermoeden van migratie van positief controle DNA (een kort synthetisch enkelstrengig DNA fragment) tussen wells gedurende real-time PCR runs geopperd als een hypothetische oorzaak van de positieve NTCs. Resultaten in dit hoofdstuk toonden aan dat verdamping van water optrad gedurende real-time PCR reacties, ongeacht het DNA type, het afsluitingstype van de reactieplaat en het gebruik van minerale olie als bedekkingslaag. Er werd ook aangegeven dat co-evaporatie van DNA optrad, met een schijnbare negatieve correlatie tussen de grootte van het DNA type en de intensiteit van deze co-evaporatie. Het gebruik van minerale olie als bedekkingslaag gecombineerd met plasmide DNA als kwantitatieve positieve PCR controle zorgde voor een complete afwezigheid van positieve NTCs terwijl enkel verwaarloosbare effecten werden waargenomen op de performantie van de real-time PCR.

Na het ontwikkelen van de real-time PCR test en het onder controle hebben van de contaminatieproblemen, werden twee protocols voor de extractie van (genomisch materiaal van) NoV uit voedingsmiddelen geëvalueerd op hun robuustheid en gevoeligheid. Daarenboven werd MNV-1 geëvalueerd als procescontrole in beide protocols. De evaluatie van een rechtstreeks RNA extractie protocol voor de extractie van genomisch materiaal (RNA) van NoV uit kant-en-klare levensmiddelen, werd beschreven in **hoofdstuk 4**, terwijl de evaluatie van een elutie-concentratie protocol voor de extractie van NoV vanuit zacht rood fruit geïllustreerd werd in **hoofdstuk 5**. Voor de kant-en-klare levensmiddelen werd voor een

direct RNA extractie protocol gekozen, gebruik makende van een guanidine isothiocyanaat bevattend reagens voor extractie van viraal DNA uit het voedselstaal (basis protocol genaamd TriShort), gevolgd door een eventuele concentratiestap gebruik makend van organische solventen (uitgebreid protocol genaamd TriConc). Het protocol voor de extractie van NoV uit zacht rood fruit bestond uit een alkalische elutie van NoV partikels uit het voedsel, gevolgd door een polyethyleen glycol (PEG) precipitatie en een daaropvolgende organische solvent opzuivering. In beide protocols werd vervolgens het RNA opgezuiverd, waarna dit opgezuiverd RNA werd gedetecteerd met behulp van de multiplex real-time RT-PCR beschreven in hoofdstuk 2. Beide NoV extractie methoden werden geëvalueerd naar robuustheid en gevoeligheid toe, door onderzoek naar de invloed van (1) het NoV inoculum niveau en (2) de verschillende voedseltypes op de recuperatie van NoV uit levensmiddelen.

Eerst en vooral werd een significante invloed van het NoV inoculum niveau op de recuperatie van NoV vanuit voedingsmiddelen aangetoond voor beide protocols. Hoog geconcentreerde NoV inocula konden succesvoller teruggevonden worden vergeleken met lager geconcentreerde NoV inocula vanuit penne salade, dat geselecteerd werd als typisch kant-en-klaar levensmiddel. Voor deze hoog geconcentreerde NoV inocula resulteerde het TriShort en TriConc protocol in gemiddelde recuperatie efficiënties van >1 % en 0.1 tot 10 %, respectievelijk. Recuperatie van deze laag en hoog geconcentreerde NoV inocula uit bevroren frambooskruim was mogelijk in veel gevallen en met gemiddelde recuperatie efficiënties van 10 tot 30 %.

Ten tweede kon een significante invloed van het voedseltype op de recuperatie van NoV aangetoond worden voor beide protocols. Het TriConc protocol gaf betere NoV recuperaties voor soepen, terwijl voor de TriShort en TriConc protocols gelijkaardige resultaten behaald werden voor samengestelde maaltijden en broodjes. NoV recuperatie uit het laatstvermelde voedseltype was echter problematisch. Eveneens werd een significante invloed van het zacht rood fruittype op de recuperatie efficiëntie van NoV GI en MNV-1 vastgesteld, terwijl er geen significante verschillen aangetoond konden worden voor GII NoV. Algemeen was recuperatie van NoV efficiënter en succesvoller vanuit de aardbeienpuree in vergelijking met de diepgevroren bosvruchtenmix en de verse frambozen.

Betreffende de evaluatie van MNV-1 als controle reagens, toonden resultaten van hoofdstukken 4 en 5 aan dat een voldoende hoge concentratie van MNV-1 nodig is om een correcte schatting te kunnen maken van de potentiële inhibitie van de RT-PCR of van inefficiënte virus extractie. Bij gebruik als reverse transcriptie controle of interne amplificatie controle, moet de concentratie zodanig aangepast worden dat interferentie met de kwantitatieve eigenschappen van de ontwikkelde multiplex real-time RT-PCR vermeden wordt.

Hoofdstuk 6 beschreef de screening van 75 fruitproducten (frambozen, aardbeien, kerstomaten en fruitsalades) op NoV aanwezigheid, waarbij gebruik gemaakt werd van het virus extractie protocol beschreven in hoofdstuk 5 gecombineerd met de multiplex real-time PCR geïllustreerd in hoofdstuk 2. In totaal testten 18 stalen positief voor genomisch materiaal van GI en/of GII NoV ondanks een goede bacteriologische kwaliteit. Het niveau van gedetecteerde NoV genomische kopijen lag tussen 2.5 en 5.0 logs per 10 gram fruitstaal. NoV GI en/of GII werden gevonden in 4/10, 7/30, 6/20 en 1/15 van de geteste frambozen, kerstomaten, aardbeien en fruitsaladestalen, respectievelijk. Bevestiging van de positieve real-time PCR resultaten door het sequeneren van genotyperingsregio's in het NoV genoom was echter niet mogelijk. De vraag of deze onverwacht hoge aantallen positieve NoV resultaten al dan niet beschouwd moeten worden als een risico voor de volksgezondheid werd gesteld en bediscussieerd.

Als conclusie kan gesteld worden dat in dit doctoraat methodes voor detectie van NoV uit zacht rood fruit en uit kant-en-klare levensmiddelen ontwikkeld en geëvalueerd werden naar robuustheid en gevoeligheid toe. Voor detectie van NoV uit zacht rood fruit en uit kant-en-klare levensmiddelen werden respectievelijk een elutie-precipitatie protocol en een direct RNA extractie protocol gecombineerd met een geoptimaliseerde multiplex real-time RT-PCR test. Dit leidde tot protocols voor detectie van NoV in levensmiddelen met een detectielimiet van ~10^4 genomische kopieën per 10 gram levensmiddel. Bovendien werd MNV-1 succesvol geëvalueerd als controle reagens en suggesties werden geopperd omtrent correct gebruik ervan. Desondanks toonde dit doctoraat ook aan dat gebruik van deze protocols ook vragen met zich meebrengt. De interpretatie van detectie van NoV met behulp van moleculaire technieken bleek niet eenvoudig, in het bijzonder naar de volksgezondheid toe.

Reference list

REFERENCE LIST

Abadias, M., Usall, J., Anguera, M., Solsona, C., and Vinas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. International Journal of Food Microbiology 123, 121-129.

Abd, E.G.K.H., El Sokkary, M.A., Kheira, S.M., Salazar, A.M., Yates, M.V., Chen, W., and Mulchandani, A., 2004. Combined immunomagnetic separation-molecular beacon-reverse transcription-PCR assay for detection of hepatitis A virus from environmental samples. Applied and Environmental Microbiology 70, 4371-4374.

Abolmaaty, A., Gu, W., Witkowsky, R., and Levin, R.E., 2007. The use of activated charcoal for the removal of PCR inhibitors from oyster samples. Journal of Microbiological Methods 68, 349-352.

Abubakar, I., Irvine, L., Aldus, C.M., Wyatt, G.M., Fordham, R., Schelenz, S., Shepstone, L., Howe, A., Peck, M., and Hunter, P.R., 2007. A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. Health Technology Assessment 11, 1-216.

Afonina, I.A., Reed, M.W., Lusby, E., Shishkina, I.G., and Belousov, Y.S., 2002. Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. Biotechniques 32, 940-949.

Anderson, A.D., Garrett, V.D., Sobel, J., Monroe, S.S., Fankhauser, R.L., Schwab, K.J., Bresee, J.S., Mead, P.S., Higgins, C., Campana, J., and Glass, R.I., 2001. Multistate outbreak of Norwalk-like virus gastroenteritis associated with a common caterer. American Journal of Epidemiology 154, 1013-1019.

Anderson, A.D., Heryford, A.G., Sarisky, J.P., Higgins, C., Monroe, S.S., Beard, R.S., Newport, C.M., Cashdollar, J.L., Fout, G.S., and Robbins, D.E., 2003. A waterborne outbreak of Norwalk like virus among snowmobilers (Wyoming, 2001). The Journal of infectious diseases 187, 303-306.

Ando, T., Monroe, S.S., Gentsch, J.R., Jin, Q., Lewis, D.C., and Glass, R.I., 1995. Detection and Differentiation of Antigenically Distinct Small Round-Structured Viruses (Norwalk-Like Viruses) by Reverse Transcription Pcr and Southern Hybridization. Journal of Clinical Microbiology 33, 64-71.

Ando, T., Noel, J.S., and Fankhauser, R.L., 2000. Genetic classification of "Norwalk-like viruses". Journal of Infectious Diseases 181, S336-S348.

Anonymous, 2002. Microbiology of food and animal feeding stuffs. Polymerase chain reaction (PCR) for the detection of food-borne pathogens. General method specific requirements (EN ISO 22174). International Organization for Standardisation, Geneva, Switzerland.

Anonymous, 2007a. The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2005. The EFSA Journal 94, 221-223.

Anonymous, 2007b. The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2006. The EFSA Journal 130, 261-264.

Anonymous, 2008. Viruses in food: scientific advice to support risk management activities. Microbiological risk assessment series 13, 1-79.

Anonymous, 2009a. Food-borne outbreaks in the European Union in 2007. The EFSA Journal 271, 18-27.

Anonymous, 2009b. Surveillance for Foodborne Disease Outbreaks--- United States, 2006. Morbidity and Mortality Weekly Report 58, 609-615.

Anonymous, 2010a. Surveillance for Foodborne Disease Outbreaks--- United States, 2007. Morbidity and Mortality Weekly Report 59, 973-979.

Anonymous, 2010b. The community summary report on trends and sources of zoonoses and zoonotic agents and foodborne outbreaks in the European Union in 2008. The EFSA Journal 1496, 233-269.

Appleton, H., 1987. Small round viruses: classification and role in food-borne infections. Ciba Foundation symposium 128, 108-125.

Appleton, H. and Pereira, M.S., 1977. A possible virus aetiology in outbreaks of food-poisoning from cockles. The Lancet 309, 780-781.

Arita, M., Ling, H., Yan, D.M., Nishimura, Y., Yoshida, H., Wakita, T., and Shimizu, H., 2009. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) system for a highly sensitive detection of enterovirus in the stool samples of acute flaccid paralysis cases. BMC Infectious Diseases 9.

Arnal, C., Ferre-Aubineau, V., Besse, B., Mignotte, B., Schwartzbrod, L., and Billaudel, S., 1999. Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A virus amplification. Journal of Virological Methods 77, 17-26.

Asanaka, M., Atmar, R.L., Ruvolo, V., Crawford, S.E., Neill, F.H., and Estes, M.K., 2005. Replication and packaging of Norwalk virus RNA in cultured mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 102, 10327-10332.

Atkinson, W., Wolfe, C., Humiston, S., and Nelson, R., 2000. Epidemiology and prevention of vaccine-preventable diseases.

Atmar, R.L., 2006. Molecular methods of virus detection in foods. Viruses in Foods 121-149.

Atmar, R.L., Neill, F.H., Romalde, J.L., Le Guyader, F., Woodley, C.M., Metcalf, T.G., and Estes, M.K., 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. Applied and Environmental Microbiology 61, 3014-3018.

Atmar, R.L., Neill, F.H., Woodley, C.M., Manger, R., Fout, G.S., Burkhardt, W., Leja, L., McGovern, E.R., Guyader, F.L., and Metcalf, T.G., 1996. Collaborative evaluation of a method for the detection of Norwalk virus in shellfish tissues by PCR. Applied and Environmental Microbiology 62, 254-258.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., and Graham, D.Y., 2008. Norwalk virus shedding after experimental human infection. Emerging Infectious Diseases 14, 1553-1557.

Bae, J. and Schwab, K.J., 2008. Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. Applied and Environmental Microbiology 74, 477-484.

Baert, L., Debevere, J., and Uyttendaele, M., 2009a. The efficacy of preservation methods to inactivate foodborne viruses. International Journal of Food Microbiology 131, 83-94.

Baert, L., Uyttendaele, M., and Debevere, J., 2007. Evaluation of two viral extraction methods for the detection of human noroviruses in shellfish with conventional and real-time reverse transcriptase PCR. Letters in Applied Microbiology 44, 106-111.

Baert, L., Uyttendaele, M., and Debevere, J., 2008a. Evaluation of viral extraction methods on a broad range of Ready-To-Eat foods with conventional and real-time RT-PCR for Norovirus GII detection. International Journal of Food Microbiology 123, 101-108.

Baert, L., Uyttendaele, M., Stals, A., Van Coillie, E., Dierick, K., Debevere, J., and Botteldoorn, N., 2009b. Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context. Epidemiology and Infection 137, 316-325.

Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J., and Uyttendaele, M., 2008b. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Applied and Environmental Microbiology 74, 543-546.

Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J., and Uyttendaele, M., 2008c. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Applied and Environmental Microbiology 74, 543-546.

Bailey, D., Thackray, L.B., and Goodfellow, I.G., 2008. Single amino acid substitution in the murine norovirus capsid protein is sufficient for attenuation in vivo. Journal of Virology 82, 7725-7728.

Balayan, M.S., Andjaparidze, A.G., Savinskaya, S.S., Ketiladze, E.S., Braginsky, D.M., Savinov, A.P., and Poleschuk, V.F., 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 20, 23-31.

Bank-Wolf, B.R., K÷nig, M., and Thiel, H.J., 2010. Zoonotic aspects of infections with noroviruses and sapoviruses. Veterinary Microbiology 140, 204-212.

Barker, J., Vipond, I.B., and Bloomfield, S.F., 2004. Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. Journal of Hospital Infection 58, 42-49.

Barr, J.N. and Fearns, R., 2010. How RNA viruses maintain their genome integrity. Journal of General Virology 91, 1373-1387.

Battaglia, M., Pedrazzoli, P., Palermo, B., Lanza, A., Bertolini, F., Gibelli, N., Da Prada, G.A., Zambelli, A., Perotti, C., and ROBUSTELLI, D.C., 1998. Epithelial tumour cell detection and the unsolved problems of nested RT-PCR: a new sensitive one step method without false positive results. Bone Marrow Transplantation 22, 693-698.

Beld, M., Minnaar, R., Weel, J., Sol, C., Damen, M., Van Der Avoort, H., Wertheim-van Dillen, P., van Breda, A., and Boom, R., 2004. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. Journal of Clinical Microbiology 42, 3059-3064.

Beuchat, L.R., 1996. Pathogenic microorganisms associated with fresh produce. Journal of Food Protection 59, 204-216.

Beuret, C., 2004. Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. Journal of Virological Methods 115, 1-8.

Bidawid, S., Farber, J.M., and Sattar, S.A., 2000. Rapid concentration and detection of hepatitis A virus from lettuce and strawberries. Journal of Virological Methods 88, 175-185.

Bidawid, S., Farber, J.M., and Sattar, S.A., 2001. Survival of hepatitis A virus on modified atmosphere-packaged (MAP) lettuce. Food Microbiology 18, 95-102.

Bidawid, S., Malik, N., Adegbunrin, O., Sattar, S.A., and Farber, J.M., 2003. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. Journal of Virological Methods 107, 163-167.

Bishop, R., 2009. Discovery of rotavirus: Implications for Child health. Journal of Gastroenterology and Hepatology 24, S81-S85.

Bishop, R.F., Davidson, G.P., Holmes, I.H., and Ruck, B.J., 1973. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. The Lancet 302, 1281-1283.

Blaise-Boisseau, S., Collette, C.H., Guillier, L., and Perelle, S., 2010. Duplex real-time qRT-PCR for the detection of hepatitis A virus in water and raspberries using the MS2 bacteriophage as a process control. Journal of Virological Methods 166, 48-53.

Boccia, D., Guthmann, J.P., Klovstad, H., Hamid, N., Tatay, M., Ciglenecki, I., Nizou, J.Y., Nicand, E., and Guerin, P.J., 2006. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. Clinical Infectious Diseases 42, 1679-1684.

Bohaychuk, V.M., Gensler, G.E., King, R.K., Manninen, K.I., Sorensen, O., Wu, J.T., Stiles, M.E., and McMullen, L.M., 2006. Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. Journal of Food Protection 69, 2176-2182.

Bohm, S.R., Brennan, B.M., Schirmer, R., and Cabose, G., 2008. Norovirus outbreak associated with ill food-service workers - Michigan, January-February 2006 (Reprinted MMWR, vol 56, pg 1212-1216, 2007). Journal of the American Medical Association 299, 164-166.

Bok, K., Abente, E.J., Realpe-Quintero, M., Mitra, T., Sosnovtsev, S.V., Kapikian, A.Z., and Green, K.Y., 2009. Evolutionary Dynamics of GII. 4 Noroviruses over a Thirty-four Year Period. Journal of Virology, Epublished ahead of print.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheimvandillen, P.M.E., and Vandernoordaa, J., 1990. Rapid and Simple Method for Purification of Nucleic-Acids. Journal of Clinical Microbiology 28, 495-503.

Borst, A., Box, A.T.A., and Fluit, A.C., 2004. False-positive results and contamination in nucleic acid amplification assays: Suggestions for a prevent and destroy strategy. European Journal of Clinical Microbiology & Infectious Diseases 23, 289-299.

Bosch, A., 2010. Human enteric viruses in the water environment: a minireview. International Microbiology 1, 191-196.

Bouwknegt, M., Lodder-Verschoor, F., van der Poel, W.H.M., Rutjes, S.A., and de Husman Roda, A.M., 2007. Hepatitis E virus RNA in commercial porcine livers in The Netherlands. Journal of Food Protection; 70, 2889-2895.

Boxman, I.L.A., Tilburg, J.J.H.C., Loeke, N.A.J.M., Vennema, H., de Boer, E., and Koopmans, M., 2007. An efficient and rapid method for recovery of norovirus from food associated with outbreaks of gastroenteritis. Journal of Food Protection 70, 504-508.

Boxman, I.L.A., Tilburg, J.J.H.C., Te Loeke, N.A.J.M., Vennema, H., Jonker, K., de Boer, E., and Koopmans, M., 2006. Detection of noroviruses in shellfish in the Netherlands. International Journal of Food Microbiology 108, 391-396.

Brooks, H.A., Gersberg, R.M., and Dhar, A.K., 2005. Detection and quantification of hepatitis A virus in seawater via real-time RT-PCR. Journal of Virological Methods 127, 109-118.

Bruggink, L.D. and Marshall, J.A., 2010. Molecular Changes Associated with Altered Patterns of Norovirus Outbreak Epidemics in Victoria, Australia, in 2006 to 2007. Journal of Clinical Microbiology 48, 857-861.

Bull, R.A., Tanaka, M.M., and White, P.A., 2007. Norovirus recombination. Journal of General Virology 88, 3347-3359.

Burkhardt, W., Blackstone, G.M., Skilling, D., and Smith, A.W., 2002. Applied technique for increasing calicivirus detection in shellfish extracts. Journal of Applied Microbiology 93, 235-240.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29, 23-29.

Bustin, S.A. and Nolan, T., 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. Journal of Biomolecular Techniques: JBT 15, 155-166.

Butot, S., Le Guyader, F.S., Krol, J., Putallaz, T., Amoroso, R., and Sβnchez, G., 2010. Evaluation of various real-time RT-PCR assays for the detection and quantitation of human norovirus. Journal of Virological Methods 167, 90-94.

Butot, S., Putallaz, T., and Sanchez, G., 2007. Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. Applied and Environmental Microbiology 73, 186-192.

Butot, S., Putallaz, T., and Sanchez, G., 2008. Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. International Journal of Food Microbiology 126, 30-35.

Cameron, C.E., Moustafa, I.M., and Arnold, J.J., 2009. Dynamics: the missing link between structure and function of the viral RNA-dependent RNA polymerase? Current opinion in structural biology 19, 768-774.

Campos, G.S., Moreau, V.H., Bandeira, A., Barberino, G., Almeida, P.F., Amador, D.M., de Lima, M.O., and Sardi, S.I., 2008. Molecular detection and genetic diversity of norovirus in hospitalized young adults with acute gastroenteritis in Bahia, Brazil. Archives of Virology 153, 1125-1129.

Candotti, D., Temple, J., Owusu-Ofori, S., and Allain, J.P., 2004. Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. Journal of Virological Methods 118, 39-47.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., and Vinje, J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. Journal of Food Protection; 69, 2761-2765.

Carlsson, B., Lindberg, A.M., Rodriguez-Diaz, J., Hedlund, K.O., Persson, B., and Svensson, L., 2009. Quasispecies dynamics and molecular evolution of human norovirus capsid P region during chronic infection. Journal of General Virology 90, 432-441.

Carter, M., 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. Journal of Applied Microbiology 98, 1354-1380.

Casas, N., Amarita, F., and de Maranon, I.M., 2007. Evaluation of an extracting method for the detection of Hepatitis A virus in shellfish by SYBR-Green real-time RT-PCR. International Journal of Food Microbiology 120, 179-185.

Caul, E.O. and Appleton, H., 1982. The Electron Microscopical and Physical Characteristics of Small Round Human Fecal Viruses - An Interim Scheme for Classification. Journal of Medical Virology 9, 257-265.

Chamberlain, J.S., Gibbs, R.A., Rainer, J.E., Nguyen, P.N., and Thomas, C., 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Research 16, 11141-11156.

Chan, M.C.W., Sung, J.J.Y., Lam, R.K.Y., Chan, P.K.S., Lee, N.L.S., Lai, R.W.M., and Leung, W.K., 2006. Fecal viral load and norovirus-associated gastroenteritis. Emerging Infectious Diseases 12, 1278-1280.

Chapron, C.D., Ballester, N.A., Fontaine, J.H., Frades, C.N., and Margolin, A.B., 2000. Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure. Applied and Environmental Microbiology 66, 2520-2525.

Chau, T.N., Lai, S.T., Tse, C., Ng, T.K., Leung, V.K.S., Lim, W., and Ng, M.H., 2006. Epidemiology and clinical features of sporadic hepatitis E as compared with hepatitis A. The American journal of gastroenterology 101, 292-296.

Cheong, S., Lee, C., Choi, W.C., Lee, C.H., and Kim, S.J., 2009a. Concentration Method for the Detection of Enteric Viruses from Large Volumes of Foods. Journal of Food Protection 72, 2001-2005.

Cheong, S., Lee, C., Song, S.W., Choi, W.C., Lee, C.H., and Kim, S.J., 2009b. Enteric Viruses in Raw Vegetables and Groundwater Used for Irrigation in South Korea. Applied and Environmental Microbiology 75, 7745-7751.

Chiba, S., Sakuma, Y., Kogasaka, R., Akihara, M., Horino, K., Nakao, T., and Fukui, S., 1979. An outbreak of gastroenteritis associated with calicivirus in an infant home. Journal of Medical Virology 4, 249-254.

Chimonas, M.A., Vaughan, G.H., Andre, Z., Ames, J.T., Tarling, G.A., Beard, S., Widdowson, M.A., and Cramer, E., 2008. Passenger behaviors associated with norovirus infection on board a cruise ship--Alaska, May to June 2004. Journal of Travel Medicine. 15, 177-183.

Chomczynski, P. and Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform. Analytical Biochemistry 162, 156-159.

Chomczynski, P. and Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanateûphenolûchloroform extraction: twenty-something years on. Nature Protocols 1, 581-585.

Cimino, G.D., Metchette, K., Isaacs, S.T., and Zhu, Y.S., 1990. More false-positive problems. Nature 345, 773-774.

Cimino, G.D., Metchette, K.C., Tessman, J.W., Hearst, J.E., and Isaacs, S.T., 1991. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Research 19, 99-107.

Clark, B. and McKendrick, M., 2004. A review of viral gastroenteritis. Current Opinion in Infectious Diseases 17, 461-469.

Cliver, D.O., 1997. Virus transmission via food. Food Technology 51, 71-81.

Cohen, J.I., Ticehurst, J.R., Purcell, R.H., Buckler-White, A., and Baroudy, B.M., 1987. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. Journal of Virology 61, 50-59.

Comelli, H.L., Rimstad, E., Larsen, S., and Myrmel, M., 2008. Detection of norovirus genotype I.3b and II.4 in bioaccumulated blue mussels using different virus recovery methods. International Journal of Food Microbiology 127, 53-59.

Compton, J., 1991. Nucleic acid sequence-based amplification. Nature 350, 91-92.

Cook, R.F., Cook, S.J., Li, F., Montelaro, R.C., and Issel, C.J., 2002. Development of a multiplex real-time reverse transcriptase-polymerase chain reaction for equine infectious anemia virus (EIAV). Journal of Virological Methods 105, 171-179.

Costafreda, M.I., Bosch, A., and Pinto, R.M., 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Applied and Environmental Microbiology 72, 3846-3855.

Costas, L., Vilella, A., Llupia, A., Bosch, J., Jimenez de Anta, M.T., and Trilla, A., 2007. Outbreak of norovirus gastroenteritis among staff at a hospital in Barcelona, Spain, September 2007. Euro Surveillance 12.

Cotterelle, B., Drougard, C., Rolland, J., Becamel, M., Boudon, M., Pinede, S., Traoré, O., Balay, K., Pothier, P., and Espié, E., 2009. Outbreak of norovirus infection associated with the consumption of frozen raspberries, France, March 2005. Euro Surveillance 10.

Coyne, K.P., Reed, F.C., Porter, C.J., Dawson, S., Gaskell, R.M., and Radford, A.D., 2006. Recombination of feline calicivirus within an endemically infected cat colony. Journal of General Virology 87, 921-926.

Craun, G.F., Calderon, R.L., and Craun, M.F., 2005. Outbreaks associated with recreational water in the United States. International Journal of Environmental Health Research 15, 243-262.

Croci, L., Dubois, E., Cook, N., De Medici, D., Schultz, A.C., China, B., Rutjes, S.A., Hoorfar, J., and van der Poel, W.H.M., 2008. Current Methods for Extraction and Concentration of Enteric Viruses from Fresh Fruit and Vegetables: Towards International Standards. Food Analytical Methods 1, 73-84.

Croci, L., Losio, M.N., Suffredini, E., Pavoni, E., Di Pasquale, S., Fallacara, F., and Arcangeli, G., 2007. Assessment of human enteric viruses in shellfish from the northern Adriatic sea. International Journal of Food Microbiology 114, 252-257.

Cromeans, T.L., Nainan, O.V., and Margolis, H.S., 1997. Detection of hepatitis A virus RNA in oyster meat. Applied and Environmental Microbiology 63, 2460-2463.

da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M., and Le Guyader, F.S., 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups I and II. Applied and Environmental Microbiology 73, 7891-7897.

Daniels, N.A., Bergmire-Sweat, D.A., Schwab, K.J., Hendricks, K.A., Reddy, S., Rowe, S.H., Fankhauser, R.L., Monroe, S.S., Atmar, R.L., Glass, R.I., and Mead, P., 2000. A foodborne outbreak of gastroenteritis associated with Norwalk-like viruses: First molecular traceback to deli sandwiches contaminated during preparation. Journal of Infectious Diseases 181, 1467-1470.

Daughenbaugh, K.F., Fraser, C.S., Hershey, J.W.B., and Hardy, M.E., 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. The EMBO Journal 22, 2852-2859.

Daughenbaugh, K.F., Wobus, C.E., and Hardy, M.E., 2006. VPg of murine norovirus binds translation initiation factors in infected cells. Virology Journal 3, 1-7.

David, S.T., McIntyre, L., MacDougall, L., Kelly, D., Liem, S., Schallie, K., McNabb, A., Houde, A., Mueller, P., Ward, P., Trottier, Y.L., and Brassard, J., 2007. An outbreak of norovirus caused by consumption of oysters from geographically dispersed harvest sites, British Columbia, Canada, 2004. Foodborne Pathogens and Disease 4, 349-358.

Dawson, D.J., Paish, A., Staffell, L.M., Seymour, I.J., and Appleton, H., 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. Journal of Applied Microbiology 98, 203-209.

de Cal, I.W., Revilla, A., del Alamo, J.M., Roman, E., Moreno, S., and Sanchez-Fauquier, A., 2007. Evaluation of two commercial enzyme immunoassays for the detection of norovirus in faecal samples from hospitalised children with sporadic acute gastroenteritis. Clinical Microbiology and Infection 13, 341-343.

de Coster, E., de Wit, M., and Widdowson, M., 2001. Large outbreak of Norwalk-like virus in ministry staff in he Netherlands. Euro Surveillance 5, 1-2.

De Medici, D., Croci, L., Suffredini, E., and Toti, L., 2004. Reverse transcription-booster PCR for detection of noroviruses in shellfish. Applied and Environmental Microbiology 70, 6329-6332.

de Paula, V.S., Gaspar, A.M.C., and Villar, L.M., 2010. Optimization of Methods for Detecting Hepatitis A Virus in Food. Food and Environmental Virology 2, 47-52.

de Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., and Teunis, P.F.M., 2009. Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. Applied and Environmental Microbiology 75, 1050-1057.

de Roda Husman, A.M., Lodder-Verschoor, F., van den Berg, H.H.J.L., Le Guyader, F.S., Van Pelt, H., van der Poel, W.H.M., and Rutjes, S.A., 2007. Rapid virus detection procedure for molecular tracing of shellfish associated with disease outbreaks. Journal of Food Protection; 70, 967-974.

De Wit, M.A.S., Widdowson, M.A., Vennema, H., de Bruin, E., Fernandes, T., and Koopmans, M., 2007. Large outbreak of norovirus: The baker who should have known better. Journal of Infection 55, 188-193.

Dedman, D., Laurichesse, H., Caul, E.O., and Wall, P.G., 1998. Surveillance of small round structured virus (SRSV) infection in England and Wales, 1990-5. Epidemiology and Infection 121, 139-149.

DeLeon, R., Matsui, S.M., Baric, R.S., Herrmann, J.E., Blacklow, N.R., Greenberg, H.B., and Sobsey, M.D., 1992. Detection of Norwalk Virus in Stool Specimens by Reverse Transcriptase-Polymerase Chain-Reaction and Nonradioactive Oligoprobes. Journal of Clinical Microbiology 30, 3151-3157.

Demeke, T. and Jenkins, G.R., 2010. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Analytical and Bioanalytical Chemistry 396, 1977-1990.

Dennehy, P.H., 2000. Transmission of rotavirus and other enteric pathogens in the home. The Pediatric Infectious Disease Journal 19, S103-S105.

Depoortere, E. and Takkinen, J., 2006. Coordinated European actions to prevent and control norovirus outbreaks on cruise ships. Euro Surveillance 11.

Di Martino, B. and Marsilio, F., 2010. Feline calicivirus VP2 is involved in the self-assembly of the capsid protein into virus-like particles. Research in Veterinary Science 89, 279-281.

Dingle, K.E., Crook, D., and Jeffery, K., 2004. Stable and noncompetitive RNA internal control for routine clinical diagnostic reverse transcription-PCR. Journal of Clinical Microbiology 42, 1003-1011.

Dippold, L., Lee, R., Selman, C., Monroe, S., and Henry, C., 2003. A gastroenteritis outbreak due to norovirus associated with a Colorado hotel. Journal of Environmental Health 66, 13-7, 26.

Dix, A.B. and Jaykus, L.A., 1998. Virion concentration method for the detection of human enteric viruses in extracts of hard-shelled clams. Journal of Food Protection; 61, 458-465.

Dolin, R., Treanor, J.J., and Madore, H.P., 1987. Novel agents of viral enteritis in humans. The Journal of infectious diseases 155, 365-376.

Dominguez, A., Torner, N., Ruiz, L., Martinez, A., Barrabeig, I., Camps, N., Godoy, P., Minguell, S., Parron, I., Pumares, A., Sala, M.R., Bartolome, R., Perez, U., de Simon, M., Montava, R., and Buesa, J., 2008. Aetiology and epidemiology of viral gastroenteritis outbreaks in Catalonia (Spain) in 2004-2005. Journal of Clinical Virology 43, 126-131.

Donaldson, E.F., Lindesmith, L.C., LoBue, A.D., and Baric, R.S., 2008. Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. Immunological reviews 225, 190-211.

Donaldson, E.F., Lindesmith, L.C., LoBue, A.D., and Baric, R.S., 2010. Viral shape-shifting: norovirus evasion of the human immune system. Nature Reviews Microbiology 8, 231-241.

Donia, D., Bonanni, E., Diaco, L., and Divizia, M., 2010. Statistical correlation between enterovirus genome copy numbers and infectious viral particles in wastewater samples. Letters in Applied Microbiology 50, 237-240.

Donia, D., Divizia, M., and Pana, A., 2005. Use of armored RNA as a standard to construct a calibration curve for real-time RT-PCR. Journal of Virological Methods 126, 157-163.

Dove, W., Cunliffe, N.A., Gondwe, J.S., Broadhead, R.L., Molyneux, M.E., Nakagomi, O., and Hart, C.A., 2005. Detection and characterization of human caliciviruses in hospitalized children with acute gastroenteritis in Blantyre, Malawi. Journal of Medical Virology 77, 522-527.

Doyle, M.P. and Erickson, M.C., 2008. Summer meeting 2007 - the problems with fresh produce: an overview. Journal of Applied Microbiology 105, 317-330.

Doyle, T.J., Stark, L., Hammond, R., and Hopkins, R.S., 2009. Outbreaks of noroviral gastroenteritis in Florida, 2006-2007. Epidemiology and Infection 137, 617-625.

Dreier, J., Stormer, M., and Kleesiek, K., 2005. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. Journal of Clinical Microbiology 43, 4551-4557.

Dubois, E., Agier, C., Traore, O., Hennechart, C., Merle, G., Cruciere, C., and Laveran, H., 2002. Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase-polymerase chain reaction or cell culture. Journal of Food Protection 65, 1962-1969.

Dubois, E., Hennechart, C., Merle, G., Burger, C., Hmila, N., Ruelle, S., Perelle, S., and Ferre, V., 2007. Detection and quantification by real-time RT PCR of hepatitis A virus from inoculated tap waters, salad vegetables, and soft fruits: Characterization of the method performances. International Journal of Food Microbiology 117, 141-149.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P.G., and Estes, M.K., 2004. Laboratory efforts to cultivate noroviruses. Journal of General Virology 85, 79-87.

Edwards, M.C. and Gibbs, R.A., 1994. Multiplex PCR - Advantages, Development, and Applications. Pcr-Methods and Applications 3, S65-S75.

Escobar-Herrera, J., Cancio, C., Guzman, G.I., Villegas-Sepulveda, N., Estrada-Garcia, T., Garcia-Lozano, H., Gomez-Santiago, F., and Gutierrez-Escolano, A.L., 2006. Construction of an

internal RT-PCR standard control for the detection of human caliciviruses in stool. Journal of Virological Methods 137, 334-338.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., and Smith, T.F., 2006. Real-time PCR in clinical microbiology: Applications for a routine laboratory testing. Clinical Microbiology Reviews 19, 165-256.

Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelso, C., and Muller, L., 2010. Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. Eurosurveillance 15, 2-4.

Ettayebi, K. and Hardy, M.E., 2003. Norwalk Virus Nonstructural Protein p48 Forms a Complex with the SNARE Regulator VAP-A and Prevents Cell Surface Expression of Vesicular Stomatitis Virus G Protein. The Journal of Virology 77, 11790-11797.

Fahy, E., Kwoh, D.Y., and Gingeras, T.R., 1991. Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. Genome Research 1, 25-33.

Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S.B., Bottiger, B., and Molbak, K., 2005. Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. Euro Surveillance 10.

Fankhauser, R.L., Monroe, S.S., Noel, J.S., Humphrey, C.D., Bresee, J.S., Parashar, U.D., Ando, T., and Glass, R.I., 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. Journal of Infectious Diseases 186, 1-7.

Feinstone, S.M., Kapikian, A.Z., and Purcell, R.H., 1973. Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. Science 182, 1026-1028.

Flamm, R.K., Hinrichs, D.J., and Thomashow, M.F., 1984. Introduction of Pam-Beta-1 Into Listeria-Monocytogenes by Conjugation and Homology Between Native L-Monocytogenes Plasmids. Infection and Immunity 44, 157-161.

Fodor, T., Reisberg, C., Hershey, H.A., and Berkowitz, H., 1970. Food poisoning occurrences in New York City, 1969. Public Health Reports 85, 1013-1018.

Fretz, R., Svoboda, P., Luthi, T.M., Tanner, M., and Baumgartner, A., 2005. Outbreaks of gastroenteritis due to infections with Norovirus in Switzerland, 2001-2003. Epidemiology and Infection 133, 429-437.

Friedman, D.S., Heisey-Grove, D., Argyros, F., Berl, E., Nsubuga, J., Stiles, T., Fontana, J., Beard, R.S., Monroe, S., McGrath, M.E., Sutherby, H., Dicker, R.C., DeMaria, A., and Matyas, B.T., 2005. An outbreak of norovirus gastroenteritis associated with wedding cakes. Epidemiology and Infection 133, 1057-1063.

Fukuda, S., Sasaki, Y., Kuwayama, M., and Miyazaki, K., 2007. Simultaneous detection and genogroup-screening test for norovirus genogroups I and II from fecal specimens in single tube by reverse transcription-loop-mediated isothermal amplification assay. Microbiology and Immunology 51, 547-550.

Fukuda, S., Takao, S., Kuwayama, M., Shimazu, Y., and Miyazaki, K., 2006. Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop-mediated isothermal amplification assay. Journal of Clinical Microbiology 44, 1376-1381.

Fumian, T.M., Leite, J.P.G., Marin, V.A., and Miagostovich, M.P., 2009. A rapid procedure for detecting noroviruses from cheese and fresh lettuce. Journal of Virological Methods 155, 39-43.

Gallimore, C.I., Barreiros, M.A.B., Brown, D.W.G., Nascimento, J.P., and Leite, J.P.G., 2004a. Noroviruses associated with acute gastroenteritis in a children's day care facility in Rio de Janeiro, Brazil. Brazilian Journal of Medical and Biological Research 37, 321-326.

Gallimore, C.I., Cubitt, D., Du Plessis, N., and Gray, J.J., 2004b. Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis. Journal of Clinical Microbiology 42, 2271-2274.

Gallimore, C.I., Pipkin, C., Shrimpton, H., Green, A.D., Pickford, Y., McCartney, C., Sutherland, G., Brown, D.W.G., and Gray, J.J., 2005. Detection of multiple enteric virus strains within a foodborne outbreak of gastroenteritis: an indication of the source of contamination. Epidemiology and Infection 133, 41-47.

Gallimore, C.I., Taylor, C., Gennery, A.R., Cant, A.J., Galloway, A., Xerry, J., Adigwe, J., and Gray, J.J., 2008. Contamination of the Hospital Environment with Gastroenteric Viruses: Comparison of Two Paediatric Wards over a Winter Season. J.Clin.Microbiol. 46, 3112-3115.

Gangarosa, E.J. and Donadio, J.A., 1970. Surveillance of Foodborne Disease in the United States. A Comparison of Data: 1968-1969. The Journal of infectious diseases 122, 354-358.

Glass, P.J., White, L.J., Ball, J.M., Leparc-Goffart, I., Hardy, M.E., and Estes, M.K., 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. Journal of Virology 74, 6581-6591.

Glass, R.I., Parashar, U.D., and Estes, M.K., 2009. Norovirus Gastroenteritis. New England Journal of Medicine 361, 1776-1785.

Glasscock, S., Welch, J., Dailer, J., Elmer, W., Kline, K., del Rosario, M., Myers, M., Buckel, T., Cvetnick, E., Myers, R., Johnson, A., Rosen, B.I., Perry, B., and Hall, A., 2007. Multistate outbreak of norovirus gastroenteritis among attendees at a family reunion--Grant County, West Virginia, October 2006. Morbidity and Mortality Weekly Report 56, 673-678.

Godoy, P., Izcara, J., Bartolome, R., Bach, P., Escobar, A., Pal, M., Codina, G., Torres, J., and Dominguez, A., 2005. Outbreak of food-borne Norovirus associated with the consumption of sandwiches. Medicina Clinica 124, 161-164.

Gonin, P. and Couillard, M., 2000. Genetic Diversity and Molecular Epidemiology of Norwalk Like Viruses. The Journal of infectious diseases 182, 691-697.

Goswami, B.B., Kulka, I., Ngo, D., Istafanos, P., and Cebula, T.A., 2002. A polymerase chain reaction-based method for the detection of hepatitis A virus in produce and shellfish. Journal of Food Protection 65, 393-402.

Gouvea, V., Santos, N., Timenetsky, M.D., and Estes, M.K., 1994. Identification of Norwalk Virus in Artificially Seeded Shellfish and Selected Foods. Journal of Virological Methods 48, 177-187.

Goyal, S.M., 2006. Viruses in Foods, Springer, New York.

Green, J., Gallimore, C.I., Norcott, J.P., Lewis, D., and Brown, D.W.G., 1995. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. Journal of Medical Virology 47, 392-398.

Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J., and Thiel, H.J., 2000. Taxonomy of the caliciviruses. Journal of Infectious Diseases 181, S322-S330.

Green, K.Y., Lew, J.F., Xi, J., Kapikian, A.Z., and Estes, M.K., 1993. Comparison of the Reactivities of Baculovirus-Expressed Recombinant Norwalk Virus Capsid Antigen with Those of the Native Norwalk Virus-Antigen in Serologic Assays and Some Epidemiologic Observations. Journal of Clinical Microbiology 31, 2185-2191.

Greening, G.E., 2006. Human and animal viruses in food (including taxonomy of enteric viruses). Viruses in Foods 5-42.

Greening, G.E. and Hewitt, J., 2008. Norovirus detection in shellfish using a rapid, sensitive virus recovery and Real-Time RT-PCR detection protocol. Food Analytical Methods 1, 109-118.

Gregory, J.B., Litaker, R.W., and Noble, R.T., 2006. Rapid one-step quantitative reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. Applied and Environmental Microbiology 72, 3960-3967.

Grotto, I., Huerta, M., Balicer, R.D., Halperin, T., Cohen, D., Orr, N., and Gdalevich, M., 2004. An outbreak of norovirus gastroenteritis on an Israeli military base. Infection 32, 339-343.

Guevremont, E., Brassard, J., Houde, A., Simard, C., and Trottier, Y.L., 2006. Development of an extraction and concentration procedure and comparison of RT-PCR primer systems for the detection of hepatitis A virus and norovirus GII in green onions. Journal of Virological Methods 134, 130-135.

Gundry, C.N., Vandersteen, J.G., Reed, G.H., Pryor, R.J., Chen, J., and Wittwer, C.T., 2003. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clinical Chemistry 49, 396-406.

Gunson, R.N., Bennett, S., Maclean, A., and Carman, W.F., 2008. Using multiplex real time PCR in order to streamline a routine diagnostic service. Journal of Clinical Virology 43, 372-375.

Guy, R.A., Payment, P., Krull, U.J., and Horgen, P.A., 2003. Real-time PCR for quantification of Giardia and Cryptosporidium in environmental water samples and sewage. Applied and Environmental Microbiology 69, 5178-5185.

Hafliger, D., Gilgen, M., Luthy, J., and Hubner, P., 1997. Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. International Journal of Food Microbiology 37, 27-36.

Hahn, H. and Palmenberg, A.C., 1995. Encephalomyocarditis viruses with short poly (C) tracts are more virulent than their mengovirus counterparts. Journal of Virology 69, 2697-2699.

Hamano, M., Kuzuya, M., Fujii, R., Ogura, H., and Yamada, M., 2005a. Epidemiology of acute gastroenteritis outbreaks caused by Noroviruses in Okayama, Japan. Journal of Medical Virology 77, 282-289.

Hamano, M., Kuzuya, M., Fujii, R., Ogura, H., and Yamada, M., 2005b. Epidemiology of acute gastroenteritis outbreaks caused by Noroviruses in Okayama, Japan. Journal of Medical Virology 77, 282-289.

Han, M.G., Smiley, J.R., Thomas, C., and Saif, L.J., 2004. Genetic recombination between two genotypes of genogroup III bovine noroviruses (BoNVs) and capsid sequence diversity among BoNVs and Nebraska-like bovine enteric caliciviruses. Journal of Clinical Microbiology 42, 5214-5224.

Hanning, I.B., Nutt, J.D., and Ricke, S.C., 2009. Salmonellosis Outbreaks in the United States Due to Fresh Produce: Sources and Potential Intervention Measures. Foodborne Pathogens and Disease 6, 635-648.

Hansman, G.S., Ishida, S., Yoshizumi, S., Miyoshi, M., Ikeda, T., Oka, T., and Takeda, N., 2007. Recombinant sapovirus gastroenteritis, Japan.

Hardy, M.E., 2005. Norovirus protein structure and function. Fems Microbiology Letters 253, 1-8.

He, J.W. and Jiang, S., 2005. Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Applied and Environmental Microbiology 71, 2250-2255.

Hedberg, C.W., MacDonald, K.L., and Osterholm, M.T., 1994. Changing Epidemiology of Food-Borne Disease - A Minnesota Perspective. Clinical Infectious Diseases 18, 671-682.

Herbert, T.P., Brierley, I., and Brown, T.D., 1997. Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. Journal of General Virology 78, 1033-1040.

Hernandez, F., Monge, R., Jimenez, C., and Taylor, L., 1997. Rotavirus and Hepatitis A virus in market lettuce (Latuca sativa) in Costa Rica. International Journal of Food Microbiology 37, 221-223.

Herrmann, M.G., Durtschi, J.D., Bromley, L.K., Wittwer, C.T., and Voelkerding, K.V., 2006. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clinical Chemistry 52, 494-503.

Herrmann, M.G., Durtschi, J.D., Wittwer, C.T., and Voelkerding, K.V., 2007. Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. Clinical Chemistry 53, 1544-1548.

Hewitt, J. and Greening, G.E., 2004. Survival and persistence of norovirus, hepatitis A virus, and feline calicivirus in marinated mussels. Journal of Food Protection 67, 1743-1750.

Hietala, S.K. and Crossley, B.M., 2006. Armored RNA as virus surrogate in a real-time reverse transcriptase PCR assay proficiency panel. Journal of Clinical Microbiology 44, 67-70.

Higuchi, R., Dollinger, G., Walsh, P.S., and Griffith, R., 1992. Simultaneous amplification and detection of specific DNA sequences. Bio/Technology 10, 413-417.

Higuchi, R., Fockler, C., Dollinger, G., and Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Nature Biotechnology 11, 1026-1030.

Hirakata, Y., Arisawa, K., Nishio, O., and Nakagomi, O., 2005. Multiprefectural spread of gastroenteritis outbreaks attributable to a single genogroup II norovirus strain from a tourist restaurant in Nagasaki, Japan. Journal of Clinical Microbiology 43, 1093-1098.

Hjertqvist, M., Johansson, A., Svensson, N., Abom, P.E., Magnusson, C., Olsson, M., Hedlund, K.O., and Andersson, Y., 2006. Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June-August 2006. Euro Surveillance 11.

Hoehne, M. and Schreier, E., 2006. Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3 '-minor groove binder-DNA probe. BMC Infectious Diseases 6.

Hogbom, M., Jager, K., Robel, I., Unge, T., and Rohayem, J., 2009. The active form of the norovirus RNA-dependent RNA polymerase is a homodimer with cooperative activity. Journal of General Virology 90, 281-291.

Hoge Gezondheidsraad, 2010. Viruses and food. HGR nr.836

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3'exonuclease activity of Thermus aquaticus DNA polymerase. Proceedings of the National Academy of Sciences of the United States of America 88, 7276-7280.

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A., and Fach, P., 2003. Making internal amplification control mandatory for diagnostic PCR. Journal of Clinical Microbiology 41, 5835-5385.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., and Fach, P., 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. Journal of Clinical Microbiology 42, 1863-1868.

Horman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C.H., Torvela, N., Heikinheimo, A., and Hanninen, M.L., 2004. Campylobacter spp., Giardia spp., Cryptosporidium noroviruses and indicator organisms in surface water in southwestern Finland, 2000-2001. Applied and Environmental Microbiology 70, 87-95.

Houde, A., Leblanc, D., Poitras, E., Ward, P., Brassard, J., Simard, C., and Trottier, Y.L., 2006. Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. Journal of Virological Methods 135, 163-172.

Hutin, Y.J.F., Pool, V., Cramer, E.H., Nainan, O.V., Weth, J., Williams, I.T., Goldstein, S.T., Gensheimer, K.F., Bell, B.P., Shapiro, C.N., Alter, M.J., and Margolis, H.S., 1999. A multistate, foodborne outbreak of hepatitis A. New England Journal of Medicine 340, 595-602.

Hutson, A.M., Atmar, R.L., and Estes, M.K., 2004. Norovirus disease: changing epidemiology and host susceptibility factors. Trends in Microbiology 12, 279-287.

Iijima, Y., Tanaka, S., and Ohishi, H., 2008. Multiple outbreaks of gastroenteritis due to a single strain of genotype II/4 Norovirus in Kobe, Japan, 2006: risk factors for norovirus spread in health care settings. Japanese Journal of Infectious Diseases 61, 419-422.

Ishii, T., Sootome, H., Shan, L., and Yamashita, K., 2007. Validation of universal conditions for duplex quantitative reverse transcription polymerase chain reaction assays. Analytical Biochemistry 362, 201-212.

Jean, J., D'Souza, D.H., and Jaykus, L.A., 2004. Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. Applied and Environmental Microbiology 70, 6603-6610.

Jenkins, P., Greene, S., Davis, J.P., Archer, J.R., Hoang-Johnson, D., Quinn, M., Duncan, P., Johnson, G., Rosen, B.I., Smith, P., Reddy, V., Schlegelmilch, J., Pendarvis, J., Donovan, M., Gunn, J.E., Barry, M.A., Davies, M., Vinje, J., Widdowson, M.A., Moore, Z., Schaffzin, J.K., and Tate, J.E., 2007. Norovirus activity - United States, 2006-2007 (Reprinted from MMWR, vol 56, pg 842, 2007). Jama-Journal of the American Medical Association 298, 1632-1634.

Jiang, X., Wang, J., Graham, D.Y., and Estes, M.K., 1992. Detection of Norwalk virus in stool by polymerase chain reaction. Journal of Clinical Microbiology 30, 2529-2534.

Joffe, M. and Robertson, A., 2001. The potential contribution of increased vegetable and fruit consumption to health gain in the European Union. Public Health Nutrition 4, 893-901.

Johannessen, G.S., Loncarevic, S., and Kruse, H., 2002. Bacteriological analysis of fresh produce in Norway. International Journal of Food Microbiology 77, 199-204.

Johansson, P.J.H., Torven, M., Hammarlund, A.C., Bjorne, U., Hedlund, K.O., and Svensson, L., 2002. Food-borne outbreak of gastroenteritis associated with genogroup I calicivirus. Journal of Clinical Microbiology 40, 794-798.

Johnston, L., 2010. Hepatitis A and B: A brief overview. SA Pharmaceutical Journal 77, 40-44.

Jones, T.H., Brassard, J., Johns, M.W., and Gagnθ, M.J., 2009. The effect of pre-treatment and sonication of centrifugal ultrafiltration devices on virus recovery. Journal of Virological Methods 161, 199-204.

Josefsson, A., Livak, K., and Gyllensten, U., 1999. Detection and quantitation of human papillomavirus by using the fluorescent 5 ' exonuclease assay. Journal of Clinical Microbiology 37, 490-496.

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., and Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. Journal of Virological Methods 131, 65-71.

Jothikumar, N., Cromeans, T.L., Sobsey, M.D., and Robertson, B.H., 2005a. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Applied and Environmental Microbiology 71, 3359-3363.

Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D.N., Hill, V.R., and Vinje, J., 2005b. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Applied and Environmental Microbiology 71, 1870-1875.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., and Katayama, K., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. Journal of Clinical Microbiology 41, 1548-1557.

Kageyama, T., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Kojima, S., Takai, R., Oka, T., Takeda, N., and Katayama, K., 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. Journal of Clinical Microbiology 42, 2988-2995.

Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R., and Chanock, R.M., 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. Journal of Virology 10, 1075-1081.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J., and Virgin IV, H.W., 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science's STKE 299, 1575-1578.

Katayama, H., Shimasaki, A., and Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. Applied and Environmental Microbiology 68, 1033-1039.

Ketner, G., Spencer, F., Tugendreich, S., Connelly, C., and Hieter, P., 1994. Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. Proceedings of the National Academy of Sciences of the United States of America 91, 6186-6190.

Khuroo, M.S., Teli, M.R., Skidmore, S., Sofi, M.A., and Khuroo, M.I., 1981. Incidence and severity of viral hepatitis in pregnancy. The American Journal of Medicine 70, 252-255.

Kiehl, W., Schreier, E., Twisselman, B., and Wunderle, W., 2001. Outbreak of Norwalk-like infection in Germany. Eurosurveillance 5, 1-1.

Kim, H.Y., Kwak, I.S., Hwang, I.G., and Ko, G., 2008a. Optimization of methods for detecting norovirus on various fruit. Journal of Virological Methods 153, 104-110.

Kim, S.R., Kim, D.W., Kwon, K.S., Hwang, I.G., and Oh, M.J., 2008b. Detection of norovirus in contaminated ham by reverse transcriptase-PCR and nested PCR. Food Science and Biotechnology 17, 651-654.

Kingsley, D.H., 2007. An RNA extraction protocol for shellfish-borne viruses. Journal of Virological Methods 141, 58-62.

Kingsley, D.H., Meade, G.K., and Richards, G.P., 2002. Detection of both hepatitis A virus and Norwalk-like virus in imported clams associated with food-borne illness. Applied and Environmental Microbiology 68, 3914-3918.

Kingsley, D.H. and Richards, G.P., 2001. Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. Applied and Environmental Microbiology 67, 4152-4157.

Kittigul, L., Pombubpa, K., Taweekate, Y., Yeephoo, T., Khamrin, P., and Ushijima, H., 2009. Molecular characterization of rotaviruses, noroviruses, sapovirus, and adenoviruses in patients with acute gastroenteritis in Thailand. Journal of Medical Virology 81, 345-353.

Kleiboeker, S.B., 2005. Quantitative assessment of the effect of uracil-DNA glycosylase on amplicon DNA degradation and RNA amplification in reverse transcription-PCR. Virology Journal 2, 29-37.

Klein, D., 2002. Quantification using real-time PCR technology: applications and limitations. Trends in Molecular Medicine 8, 257-260.

Kobayashi, S., Natori, K., Takeda, N., and Sakae, K., 2004. Immunomagnetic capture RT-PCR for detection of norovirus from foods implicated in foodborne outbreak. Microbiology and Immunology 48, 201-204.

Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F.B., Shinohara, M., Uchida, K., Natori, K., Takeda, N., and Katayama, K., 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. Journal of Virological Methods 100, 107-114.

Koopmans, M., 2008. Progress in understanding norovirus epidemiology. Current Opinion in Infectious Diseases 21, 544-552.

Koopmans, M. and Duizer, E., 2004. Foodborne viruses: an emerging problem. International Journal of Food Microbiology 90, 23-41.

Koopmans, M., von Bonsdorff, C.H., Vinje, J., De Medici, D., and Monroe, S., 2002. Foodborne viruses. Fems Microbiology Reviews 26, 187-205.

Krause, G., Altmann, D., Faensen, D., Porten, K., Benzler, J., Pfoch, T., Ammon, A., Kramert, M.H., and Claus, H., 2007. SurvNet electronic surveillance system for infectious disease outbreaks, Germany. Emerging Infectious Diseases 13, 1548-1555.

Kroneman, A., Harris, J., Vennema, H., Duizer, E., van Duynhoven, Y., Gray, J., Iturriza, M., Bottiger, B., Falkenhorst, G., Johnsen, C., von Bonsdorff, C.H., Maunula, L., Kuusi, M., Pothier, P., Gallay, A., Schreier, E., Koch, J., Szuecs, G., Reuter, G., Krisztalovics, K., Lynch, M., McKeown, P., Foley, B., Coughlan, S., Ruggeri, F.M., Di Bartolo, I., Vainio, K., Isakbaeva, E., Poljsak-Prijatelj, M., Grom, A.H., Bosch, A., Buesa, J., Fauquier, A.S., Hernandez-Pezzi, G., Hedlund, K.O., and Koopmans, M., 2008a. Data quality of 5 years of central norovirus outbreak reporting in the European Network for food-borne viruses. Journal of Public Health 30, 82-90.

Kroneman, A., Verhoef, L., Harris, J., Vennema, H., Duizer, E., van Duynhoven, Y., Gray, J., Iturriza, M., Bottiger, B., Falkenhorst, G., Johnsen, C., von Bonsdorff, C.H., Maunula, L., Kuusi, M., Pothier, P., Gallay, A., Schreier, E., Hohne, M., Koch, J., Szucs, G., Reuter, G., Krisztalovics, K., Lynch, M., McKeown, P., Foley, B., Coughlan, S., Ruggeri, F.M., Di Bartolo, I., Vainio, K., Isakbaeva, E., Poljsak-Prijatelj, M., Grom, A.H., Mijovski, J.Z., Bosch, A., Buesa, J., Fauquier, A.S., Hernandez-Pezzi, G., Hedlund, K.O., and Koopmans, M., 2008b. Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the Foodborne Viruses in Europe Network from 1 July 2001 to 30 June 2006. Journal of Clinical Microbiology 46, 2959-2965.

Krumbholz, A., Meerbach, A., Zell, R., Gruhn, B., Henke, A., Birch-Hirschfeld, E., and Wutzler, P., 2006. Comparison of a LightCycler-based real-time PCR for quantitation of Epstein-Barr viral load in different clinical specimens with semiquantitative PCR. Journal of Medical Virology 78, 598-607.

Kubista, M., 2004. Nucleic acid-based technologies: application amplified. Future Medicine 5, 767-773.

Kuo, H.W., Schmid, D., Jelovcan, S., Pichler, A.M., Magnet, E., Reichart, S., and Allerberger, F., 2009. A Foodborne Outbreak Due to Norovirus in Austria, 2007. Journal of Food Protection 72, 193-196.

Kurdziel, A.S., Wilkinson, N., Langton, S., and Cook, N., 2001. Survival of poliovirus on soft fruit and salad vegetables. Journal of Food Protection 64, 706-709.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., and Hedgpeth, J., 2000. Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Research 28, 655-661.

Kwoh, D.Y., Davis, G.R., Whitfield, K.M., Chappelle, H.L., DiMichele, L.J., and Gingeras, T.R., 1989. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proceedings of the National Academy of Sciences of the United States of America 86, 1173-1177.

Kwok, S. and Higuchi, R., 1989. Avoiding False Positives with Pcr. Nature 339, 237-238.

La Rosa, G., Fontana, S., Di Grazia, A., Iaconelli, M., Pourshaban, M., and Muscillo, M., 2007. Molecular identification and genetic analysis of norovirus genogroups I and II in water environments: Comparative analysis of different reverse transcription-PCR assays (vol 73, pg 4152). Applied and Environmental Microbiology 73, 6329-6329.

Lambden, P.R., Cooke, S.J., Caul, E.O., and Clarke, I.N., 1992. Cloning of noncultivatable human rotavirus by single primer amplification. Journal of Virology 66, 1817-1822.

Lamhoujeb, S., Fliss, I., Ngazoa, S.E., and Jean, J., 2008. Evaluation of the Persistence of Infectious Human Noroviruses on Food Surfaces using Molecular Beacon-Based Nucleic Acid Sequence-Based Amplification. Applied and Environmental Microbiology 74, 3349-3355.

Lamothe, G.T., Putallaz, T., Joosten, H., and Marugg, J.D., 2003. Reverse transcription-PCR analysis of bottled and natural mineral waters for the presence of noroviruses. Applied and Environmental Microbiology 69, 6541-6549.

Lampel, K.A., Orlandi, P.A., and Kornegay, L., 2000. Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. Applied and Environmental Microbiology 66, 4539-4542.

Lantz, P.G., Matsson, M., Wadstrom, T., and Radstrom, P., 1997. Removal of PCR inhibitors from human faecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR. Journal of Microbiological Methods 28, 159-167.

Lau, C.S., Wong, D.A., Tong, L.K.L., Lo, J.Y.C., Ma, A.M.C., Cheng, P.K.C., and Lim, W.W.L., 2004. High rate and changing molecular epidemiology pattern of norovirus infections in sporadic cases and outbreaks of gastroenteritis in Hong Kong. Journal of Medical Virology 73, 113-117.

Laverick, M.A., Wyn-Jones, A.P., and Carter, M.J., 2004. Quantitative RT-PCR for the enumeration of noroviruses (Norwalk-like viruses) in water and sewage. Letters in Applied Microbiology 39, 127-136.

Le Guyader, F., Miossec, L., Haugarreau, L., Dubois, E., Kopecka, H., and Pommepuy, M., 1998. RT-PCR evaluation of viral contamination in five shellfish beds over a 21-month period. Water Science and Technology 38, 45-50.

Le Guyader, F.S., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., Doyle, A., Zidane, M., Suffredini, E., Kohli, E., Maddalo, F., Monini, M., Gallay, A., Pommepuy, M., Pothier, P., and Ruggeri, F.M., 2006. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J.Clin.Microbiol. 44, 3878-3882.

Le Guyader, F.S., Le Saux, J.C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., Giraudon, H., Delmas, G., Pommepuy, M., and Pothier, P., 2008. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. Journal of Clinical Microbiology 46, 4011-4017.

Le Guyader, F.S., Mittelholzer, C., Haugarreau, L., Hedlund, K.O., Alsterlund, R., Pommepuy, M., and Svensson, L., 2004a. Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. International Journal of Food Microbiology 97, 179-186.

Le Guyader, F.S., Neill, F.H., Dubois, E., Bon, F., Loisy, F., Kohli, E., Pommepuy, M., and Atmar, R.L., 2003. A semiquantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. Int.J.Food Microbiol. 87, 107-112.

Le Guyader, F.S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M., and Atmar, R.L., 2009. Detection and quantification of noroviruses in shellfish. Applied and Environmental Microbiology 75, 618-624.

Le Guyader, F.S., Schultz, A.C., Haugarreau, L., Croci, L., Maunula, L., Duizer, E., Lodder-Verschoor, F., von Bonsdorff, C.H., Suffredini, E., Van Der Poel, W.M.M., Reymundo, R., and Koopmans, M., 2004b. Round-robin comparison of methods for the detection of human enteric viruses in lettuce. Journal of Food Protection 67, 2315-2319.

Leb, V., Stocher, M., Valentine-Thon, E., Holzl, G., Kessler, H., Stekel, H., and Berg, J., 2004. Fully automated, internally controlled quantification of hepatitis B virus DNA by real-time PCR by use of the MagNA Pure LC and LightCycler instruments. Journal of Clinical Microbiology 42, 585-590.

Lederer, I., Schmid, D., Pichler, A.M., Dapra, R., Kraler, P., Blassnig, A., Luckner-Hornischer, A., Berghold, C., and Allerberger, F., 2005. Outbreak of norovirus infections associated with consuming food from a catering company, Austria, September 2005. Euro Surveillance 10.

Lee, N., Chan, M.C.W., Wong, B., Choi, K.W., Sin, W., Lui, G., Chan, P.K.S., Lai, R.W.M., Cockram, C.S., Sung, J.J.Y., and Leung, W.K., 2007. Fecal viral concentration and diarrhea in norovirus gastroenteritis. Emerging Infectious Diseases 13, 1399-1401.

Lees, D., 2010. International Standardisation of a Method for Detection of Human Pathogenic Viruses in Molluscan Shellfish. Food and Environmental Virology 2, 146-155.

Leggitt, P.R. and Jaykus, L.A., 2000. Detection methods for human enteric viruses in representative foods. Journal of Food Protection 63, 1738-1744.

Lekanne Deprez, R.H., Fijnvandraat, A.C., Ruijter, J.M., and Moorman, A.F.M., 2002. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. Analytical Biochemistry 307, 63-69.

Leong, Y.K., Xui, O.C., and Chia, O.K., 2008. Survival of SA11 Rotavirus in Fresh Fruit Juices of Pineapple, Papaya, and Honeydew Melon. Journal of Food Protection 71, 1035-1037.

Lewis, G.D. and Metcalf, T.G., 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Applied and Environmental Microbiology 54, 1983-1988.

Li, D., Ing-juan, T., Hao-jie, L.I., U-ming, W., Ong, X.U.E., and Hang-hu, X.U.E., 2009. Comparative Study of Detection of Norovirus in Oysters by RT-PCR, RT-Seminested PCR and RT-LAMP. Food Science 12.

Liebermann, H. and Mentel, R., 1994. Quantification of adenovirus particles. Journal of Virological Methods 50, 281-291.

Lindesmith, L.C., Donaldson, E.F., LoBue, A.D., Cannon, J.L., Zheng, D.P., Vinje, J., and Baric, R.S., 2008. Mechanisms of GII. 4 norovirus persistence in human populations. PLoS Med 5, e31-e36.

Lindquist, L. and Vapalahti, O., 2008. Tick-borne encephalitis. The Lancet 371, 1861-1871.

Liu, P., Chien, Y.W., Papafragkou, E., Hsiao, H.M., Jaykus, L.A., and Moe, C., 2009. Persistence of Human Noroviruses on Food Preparation Surfaces and Human Hands. Food and Environmental Virology 1, 141-147.

Lodder, W.J. and de Roda Husman, A.M., 2005. Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. Applied and Environmental Microbiology 71, 1453-1461.

Loisy, F., Atmar, R.L., Guillon, P., Le Cann, P., Pommepuy, M., and Le Guyader, F.S., 2005. Real-time RT-PCR for norovirus screening in shellfish. Journal of Virological Methods 123, 1-7.

Longo, M.C., Berninger, M.S., and Hartley, J.L., 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 93, 125-128.

Lopman, B.A., Adak, G.K., Reacher, M.H., and Brown, D.W.G., 2003a. Two epidemiologic patterns of Norovirus outbreaks: Surveillance in England and Wales, 1992-2000. Emerging Infectious Diseases 9, 71-77.

Lopman, B.A., Reacher, M.H., van, D.Y., Hanon, F.X., Brown, D., and Koopmans, M., 2003b. Viral gastroenteritis outbreaks in Europe, 1995-2000. Emerging Infectious Diseases 9, 90-96.

Lopman, B.A., Reacher, M.H., Vipond, I.B., Sarangi, J., and Brown, D.W.G., 2004. Clinical manifestation of norovirus gastroenteritis in health care settings. Clinical Infectious Diseases 39, 318-324.

Love, D.C., Casteel, M.J., Meschke, J.S., and Sobsey, M.D., 2008. Methods for recovery of hepatitis A virus (HAV) and other viruses from processed foods and detection of HAV by nested RT-PCR and TaqMan RT-PCR. International Journal of Food Microbiology 126, 221-226.

Lowe, B., Avila, H.A., Bloom, F.R., Gleeson, M., and Kusser, W., 2003. Quantitation of gene expression in neural precursors by reverse-transcription polymerase chain reaction using self-quenched, fluorogenic primers. Analytical Biochemistry 315, 95-105.

Lowther, J.A., Avant, J.M., Gizynski, K., Rangdale, R.E., and Lees, D.N., 2010. Comparison between Quantitative Real-Time Reverse Transcription PCR Results for Norovirus in Oysters and Self-

Reported Gastroenteric Illness in Restaurant Customers. Journal of Food Protection 73, 305-311.

Ludwig, A., Adams, O., Laws, H.J., Schroten, H., and Tenenbaum, T., 2008. Quantitative detection of norovirus excretion in pediatric patients with cancer and prolonged gastroenteritis and shedding of norovirus. Journal of Medical Virology 80, 1461-1467.

Lynch, M., Painter, J., Woodruff, R., and Braden, C., 2006. Surveillance for foodborne-disease outbreaksùUnited States, 1998-2002. Morbidity and Mortality Weekly Report 55, 1-42.

Mackay, I.M., Arden, K.E., and Nitsche, A., 2002. Real-time PCR in virology. Nucleic Acids Research 30, 1292-1305.

Maguire, A.J., Green, J., Brown, D.W.G., Desselberger, U., and Gray, J.J., 1999. Molecular epidemiology of outbreaks of gastroenteritis associated with small round-structured viruses in East Anglia, United Kingdom, during the 1996-1997 season. Journal of Clinical Microbiology 37, 81-89.

Makary, P., Maunula, L., Niskanen, T., Kuusi, M., Virtanen, M., Pajunen, S., Ollgren, J., and Minh, N.N.T., 2009. Multiple norovirus outbreaks among workplace canteen users in Finland, July 2006. Epidemiology and Infection 137, 402-407.

Malorny, B., Tassios, P.T., Radstrom, P., Cook, N., Wagner, M., and Hoorfar, J., 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int.J.Food Microbiol. 83, 39-48.

Marshall, J.A., Dimitriadis, A., and Wright, P.J., 2005. Molecular and epidemiological features of norovirus-associated gastroenteritis outbreaks in Victoria, Australia in 2001. Journal of Medical Virology 75, 321-331.

Martella, V., Campolo, M., Lorusso, E., Cavicchio, P., Camero, M., Bellacicco, A.L., Decaro, N., Elia, G., Greco, G., and Corrente, M., 2007. Norovirus in captive lion cub (Panthera leo). Emerging Infectious Diseases 13, 1071-1073.

Martella, V., Lorusso, E., Decaro, N., Elia, G., Radogna, A., D'Abramo, M., Desario, C., Cavalli, A., Corrente, M., Camero, M., Germinario, C.A., Banyai, K., Di, M.B., Marsilio, F., Carmichael, L.E., and Buonavoglia, C., 2008. Detection and molecular characterization of a canine norovirus. Emerging Infectious Diseases 14, 1306-1308.

Martin, A. and Lemon, S.M., 2006. Hepatitis A virus: from discovery to vaccines. Hepatology 43, S164-S172.

Mathijs, E., Muylkens, B., Mauroy, A., Ziant, D., Delwiche, T., and Thiry, E., 2010. Experimental Evidence of Recombination in Murine Noroviruses. Journal of General Virology Article in press,

Matsui, S.M., Kim, J.P., Greenberg, H.B., Young, L.M., Smith, L.S., Lewis, T.L., Herrmann, J.E., Blacklow, N.R., Dupuis, K., and Reyes, G.R., 1993. Cloning and characterization of human astrovirus immunoreactive epitopes. Journal of Virology 67, 1712-1715.

Mattison, K., Brassard, J., Gagne, M.J., Ward, P., Houde, A., Lessard, L., Simard, C., Shukla, A., Pagotto, F., and Jones, T.H., 2009a. The feline calicivirus as a sample process control for the detection of food and waterborne RNA viruses. International Journal of Food Microbiology 132, 73-77.

Mattison, K., Grudeski, E., Auk, B., Brassard, J., Charest, H., Dust, K., Gubbay, J., Hatchette, T.F., Houde, A., and Jean, J., 2010a. Analytical performance of norovirus real-time RT-PCR detection protocols in Canadian laboratories. Journal of Clinical Virology, article in press.

Mattison, K., Grudeski, E., Auk, B., Charest, H., Drews, S.J., Fritzinger, A., Gregoricus, N., Hayward, S., Houde, A., and Lee, B.E., 2009b. Multicenter Comparison of Two Norovirus ORF2-Based Genotyping Protocols. Journal of Clinical Microbiology 47, 3927-3932.

Mattison, K., Harlow, J., Morton, V., Cook, A., Pollari, F., and Bidawid, S., 2010b. Enteric viruses in ready-to-eat packaged leafy greens. Emerging Infectious Diseases 16, 1815-1817.

Mattison, K., Shukla, A., Cook, A., Pollari, F., Friendship, R., Kelton, D., Bidawid, S., and Farber, J.M., 2007. Human noroviruses in swine and cattle. Emerging Infectious Diseases 13, 1184-1188.

Mattner, F., Sohr, D., Heim, A., Gastmeier, P., Vennema, H., and Koopmans, M., 2006. Risk groups for clinical complications of norovirus infections: an outbreak investigation. Clinical Microbiology and Infection 12, 69-74.

Maunula, L., Roivainen, M., Keranen, M., Makela, S., Soderberg, K., Summa, M., von Bonsdorff, C.H., Lappalainen, M., Korhonen, T., Kuusi, M., and Niskanen, T., 2009. Detection of Human Norovirus from Frozen Raspberries in A Cluster of Gastroenteritis Outbreaks. Eurosurveillance 14, 16-18.

Maunula, L. and von Bonsdorff, C.H., 2005. Norovirus genotypes causing gastroenteritis outbreaks in Finland 1998-2002. Journal of Clinical Virology 34, 186-194.

Mauroy, A., Scipioni, A., Matthijs, E., Miry, C., Ziant, D., Thys, C., and Thiry, E., 2008. Noroviruses and sapoviruses in pigs in Belgium. Archives of Virology 153, 1927-1931.

McIngvale, S.C., Elhanafi, D., and Drake, M.A., 2002. Optimization of reverse transcriptase PCR to detect viable Shiga-toxin-producing Escherichia coli. Applied and Environmental Microbiology 68, 799-806.

McKillip, J.L. and Drake, M., 2004. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. Journal of Food Protection 67, 823-832.

Mead, P.S., Slutsker, L., Dietz, V., Mccaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5, 607-625.

Medici, M.C., Abelli, L.A., Dodi, I., Dettori, G., and Chezzi, C., 2010. Norovirus RNA in the blood of a child with gastroenteritis and convulsionsùA case report. Journal of Clinical Virology 48, 147-149.

Medici, M.C., Morelli, A., Arcangeletti, M.C., Calderaro, A., De Conto, F., Martinelli, M., Abelli, L.A., Detorri, G., and Chezzi, C., 2008. An outbreak of norovirus infection in an Italian residential-care facility for the elderly. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infections Diseases 15, 97-100.

Mendez, I.I., Hermann, L.L., Hazelton, P.R., and Coombs, K.M., 2000. A comparative analysis of Freon substitutes in the purification of reovirus and calicivirus. Journal of Virological Methods 90, 59-67.

Menton, J.F., Kearney, K., and Morgan, J.G., 2007. Development of a real-time RT-PCR and Reverse Line probe Hybridisation assay for the routine detection and genotyping of Noroviruses in Ireland. Virology Journal 4, 94-102.

Mesquita, J.R. and Nascimento, M.S.J., 2009. A Foodborne Outbreak of Norovirus Gastroenteritis Associated with A Christmas Dinner in Porto, Portugal, December 2008. Eurosurveillance 14, 19-21.

Michen, B. and Graule, T., 2010. Isoelectric points of viruses. Journal of Applied Microbiology 109, 388-397.

Milne, S.A., Gallacher, S., Cash, P., Lees, D.N., Henshilwood, K., and Porter, A.J.R., 2007. A Sensitive and Reliable Reverse Transcriptase PCREnzyme-Linked Immunosorbent Assay for the Detection of Human Pathogenic Viruses in Bivalve Molluscs. Journal of Food Protection; 70, 1475-1482.

Mobius, P., Hotzel, H., Rassbach, A., and Kohler, H., 2008. Comparison of 13 single-round and nested PCR assays targeting IS900, ISMav2, f57 and locus 255 for detection of Mycobacterium avium subsp. paratuberculosis. Veterinary Microbiology 126, 324-333.

Molenkamp, R., van der, H.A., Schinkel, J., and Beld, M., 2007. Simultaneous detection of five different DNA targets by real-time Taqman PCR using the Roche LightCycler480: Application in viral molecular diagnostics. Journal of Virological Methods 141, 205-211.

Monis, P.T., Giglio, S., and Saint, C.P., 2005. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Analytical Biochemistry 340, 24-34.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J., and Megraud, F., 1997. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. Journal of Clinical Microbiology 35, 995-998.

Moore, C., Clark, E.M., Gallimore, C.I., Corden, S.A., Gray, J.J., and Westmoreland, D., 2004. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. Journal of Clinical Virology 29, 290-296.

Morales-Rayas, R., Wolffs, P.F.G., and Griffiths, M.W., 2009. Anion-Exchange Filtration and Real-Time PCR for the Detection of a Norovirus Surrogate in Food. Journal of Food Protection 72, 2178-2183.

Morales-Rayas, R., Wolffs, P.F.G., and Griffiths, M.W., 2010. Simultaneous separation and detection of hepatitis A virus and norovirus in produce. International Journal of Food Microbiology 139, 48-55.

Morioka, S., Sakata, T., Tamaki, A., Shioji, T., Funaki, A., Yamamoto, Y., Naka, H., Terasoma, F., Imai, K., and Matsuo, K., 2006. A food-borne norovirus outbreak at a primary school in Wakayama Prefecture. Japanese Journal of Infectious Diseases 59, 205-207.

Moriya, Y., Nakamura, T., Okamura, N., Sakaeda, T., Horinouchi, M., Tamura, T., Aoyama, N., Kasuga, M., and Okumura, K., 2006. Comparison of synthetic DNA templates with authentic cDNA templates in terms of quantification by real-time quantitative reverse transcription polymerase chain reaction. Biological & Pharmaceutical Bulletin 29, 535-538.

Morton, V., Jean, J., Farber, J., and Mattison, K., 2009. Detection of Noroviruses in Ready-To-Eat Foods by Using Carbohydrate-Coated Magnetic Beads. Applied and Environmental Microbiology 75, 4641-4643.

Mukherjee, A., Speh, D., Dyck, E., and Diez-Gonzalez, F., 2004. Preharvest evaluation of coliforms, Escherichia coli, Salmonella, and Escherichia coli O157 : H7 in organic and conventional produce grown by Minnesota farmers. Journal of Food Protection 67, 894-900.

Mullendore, J.L., Sobsey, M.D., and Carol Shieh, Y.S., 2001. Improved method for the recovery of hepatitis A virus from oysters. Journal of Virological Methods 94, 25-35.

Nayak, M.K., Chatterjee, D., Nataraju, S.M., Pativada, M., Mitra, U., Chatterjee, M.K., Saha, T.K., Sarkar, U., and Krishnan, T., 2009. A new variant of Norovirus GII. 4/2007 and inter-genotype recombinant strains of NVGII causing acute watery diarrhoea among children in Kolkata, India. Journal of Clinical Virology 45, 223-229.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., and Rashtchian, A., 2002. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Research 30, e37-e43.

Nenonen, N.P., Hannoun, C., Olsson, M.B., and Bergstrom, T., 2009. Molecular analysis of an oyster-related norovirus outbreak. Journal of Clinical Virology 45, 105-108.

Ng, T.L., Chan, P.P., Phua, T.H., Loh, J.P., Yip, R., Wong, C., Liaw, C.W., Tan, B.H., Chiew, K.T., Chua, S.B., Lim, S., Ooi, P.L., Chew, S.K., and Goh, K.T., 2005. Oyster-associated outbreaks of Norovirus gastroenteritis in Singapore. Journal of Infection 51, 413-418.

Niesters, H.G., 2002. Clinical virology in real time. Journal of Clinical Virology 25 (Suppl 3), S3-S12.

Nilsson, M., Hedlund, K.O., Thorhagen, M., Larson, G., Johansen, K., Ekspong, A., and Svensson, L., 2003. Evolution of human calicivirus RNA in vivo: accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. Journal of Virology 77, 13117-13124.

Nishida, T., Kimura, H., Saitoh, M., Shinohara, M., Kato, M., Fukuda, S., Munemura, T., Mikami, T., Kawamoto, A., Akiyama, M., Kato, Y., Nishi, K., Kozawa, K., and Nishio, O., 2003. Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. Applied and Environmental Microbiology 69, 5782-5786.

Nishida, T., Nishio, O., Kato, M., Chuma, T., Kato, H., Iwata, H., and Kimura, H., 2007. Genotyping and quantitation of noroviruses in oysters from Two Distinct Sea areas in Japan. Microbiology and Immunology 51, 177-184.

Nocker, A. and Camper, A.K., 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. Fems Microbiology Letters 291, 137-142.

Nocker, A., Cheung, C.Y., and Camper, A.K., 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. Journal of Microbiological Methods 67, 310-320.

Noda, M., Fukuda, S., and Nishio, O., 2008. Statistical analysis of attack rate in norovirus foodborne outbreaks. International Journal of Food Microbiology 122, 216-220.

Noel, J.S., Ando, T., Leite, J.P., Green, K.Y., Dingle, K.E., Estes, M.K., Seto, Y., Monroe, S.S., and Glass, R.I., 1997. Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. Journal of Medical Virology 53, 372-383.

Nordgren, J., Bucardo, F., Dienus, O., Svensson, L., and Lindgren, P.E., 2008. Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens. Journal of Clinical Microbiology 46, 164-170.

Nordgren, J., Bucardo, F., Svensson, L., and Lindgren, P.E., 2010a. Novel Light-Upon-Extension Real-Time PCR Assay for Simultaneous Detection, Quantification, and Genogrouping of Group A Rotavirus. Journal of Clinical Microbiology 48, 1859-1864.

Nordgren, J., Kindberg, E., Lindgren, P.E., Matussek, A., and Svensson, L., 2010b. Norovirus Gastroenteritis Outbreak with a Secretor-independent Susceptibility Pattern, Sweden. Emerging Infectious Diseases 16, 81-87.

Nothdurft, H.D., 2008. Hepatitis A vaccines. Expert Review of Vaccines 7, 535-545.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28, e63-1-e63-7.

Nuanualsuwan, S. and Cliver, D.O., 2002. Pretreatment to avoid positive RT-PCR results with inactivated viruses. Journal of Virological Methods 104, 217-225.

Nyrén, P., 2007. The History of Pyrosequencing. Methods in molecular biology (Clifton, NJ) 373, 1-14.

O'Brien, S.J., Gillespie, I.A., Sivanesan, M.A., Elson, R., Hughes, C., and Adak, G.K., 2006. Publication bias in foodborne outbreaks of infectious intestinal disease and its implications for evidence-based food policy. England and Wales 1992-2003. Epidemiology and Infection 134, 667-674.

O'Brien, S.J., Mitchell, R.T., Gillespie, I.A., and Adak, G.K., 2000. Microbiological status of ready-to-eat fruit and vegetables. Discussion paper: advisory committee on the microbiological of food. 476, 1-34.

O'Neill, H.J., McCaughey, C., Wyatt, D.E., Mitchell, F., and Coyle, P.V., 2001. Gastroenteritis outbreaks associated with Norwalk-like viruses and their investigation by nested RT-PCR. BMC microbiology 1, 14-22.

Ohwaki, K., Nagashima, H., Aoki, M., Aoki, H., and Yano, E., 2009. A Foodborne Norovirus Outbreak at a Hospital and an Attached Long-Term Care Facility. Japanese Journal of Infectious Diseases 62, 450-454.

Oikarinen, S., Tauriainen, S., Viskari, H., Simell, O., Knip, M., Virtanen, S., and Hy÷ty, H., 2009. PCR inhibition in stool samples in relation to age of infants. Journal of Clinical Virology 44, 211-214.

Oliver, S.L., Asobayire, E., Dastjerdi, A.M., and Bridger, J.C., 2006. Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae. Virology 350, 240-250.

Oogane, T., Hirata, A., Funatogawa, K., Kobayashi, K., Sato, T., and Kimura, H., 2008. Food Poisoning Outbreak Caused by Norovirus GII/4 in School Lunch, Tochigi Prefecture, Japan. Japanese Journal of Infectious Diseases 61, 423-424.

Orru, G., Masia, G., Orru, G., Romano, L., Piras, V., and Coppola, R.C., 2004. Detection and quantitation of hepatitis E virus in human faeces by real-time quantitative PCR. Journal of Virological Methods 118, 77-82.

Oszmianski, J. and Wojdylo, A., 2009. Comparative study of phenolic content and antioxidant activity of strawberry puree, clear, and cloudy juices. European Food Research and Technology 228, 623-631.

Ozawa, K., Oka, T., Takeda, N., and Hansman, G.S., 2007. Norovirus infections in symptomatic and asymptomatic food handlers in Japan. Journal of Clinical Microbiology 45, 3996-4005.

Pagotto, F., Corneau, N., Mattison, K., and Bidawid, S., 2008. Development of a DNA Microarray for the Simultaneous Detection and Genotyping of Noroviruses. Journal of Food Protection 71, 1434-1441.

Palmer, S., Brown, D., and Morgan, D., 2005. Early qualitative risk assessment of the emerging zoonotic potential of animal diseases. British Medical Journal 331, 1256-1260.

Pan, Y. and Breidt Jr, F., 2007. Enumeration of viable Listeria monocytogenes cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Applied and Environmental Microbiology 73, 8028-8031.

Panda, S.K., Thakral, D., and Rehman, S., 2007. Hepatitis E virus. Reviews in Medical Virology 17, 151-180.

Pang, J., Modlin, J., and Yolken, R., 1992. Use of Modified Nucleotides and Uracil-Dna Glycosylase (Ung) for the Control of Contamination in the Pcr-Based Amplification of Rna. Molecular and Cellular Probes 6, 251-256.

Pang, X.L., Lee, B., Chui, L., Preiksaitis, J.K., and Monroe, S.S., 2004. Evaluation and validation of real-time reverse transcription-PCR assay using the LightCycler system for detection and quantitation of norovirus. Journal of Clinical Microbiology 42, 4679-4685.

Pang, X.L.L., Preiksaitis, J.K., and Lee, B., 2005. Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. Journal of Clinical Virology 33, 168-171.

Papafragkou, E., Plante, M., Mattison, K., Bidawid, S., Karthikeyan, K., Farber, J.M., and Jaykus, L.A., 2008. Rapid and sensitive detection of hepatitis A virus in representative food matrices. Journal of Virological Methods 147, 177-187.

Parashar, U.D., Dow, L., Fankhauser, R.L., Humphrey, C.D., Miller, J., Ando, T., Williams, K.S., Eddy, C.R., Noel, J.S., Ingram, T., Bresee, J.S., Monroe, S.S., and Glass, R.I., 1998. An outbreak of viral gastroenteritis associated with consumption of sandwiches: implications for the control of transmission by food handlers. Epidemiology and Infection 121, 615-621.

Parasidis, T., Divari, E., Fatouros, N., and Vantarakis, A., 2007. Outbreak of acute gastroenteritis in Greece during a school excursion, April 2007. Euro Surveillance 12, E0705-E0707.

Park, Y., Cho, Y.H., Jee, Y., and Ko, G., 2008. Immunomagnetic separation combined with real-time reverse transcriptase PCR assays for detection of norovirus in contaminated food. Applied and Environmental Microbiology 74, 4226-4230.

Parshionikar, S., Laseke, I., and Fout, G.S., 2010. Use of Propidium Monoazide in Reverse Transcriptase PCR To Distinguish between Infectious and Noninfectious Enteric Viruses in Water Samples. Applied and Environmental Microbiology 76, 4318-4326.

Parshionikar, S.U., Cashdollar, J., and Fout, G.S., 2004. Development of homologous viral internal controls for use in RT-PCR assays of waterborne enteric viruses. Journal of Virological Methods 121, 39-48.

Payne, K., Hall, M., Lutzke, M., Armstrong, C., and King, J., 2006. Multisite outbreak of norovirus associated with a franchise restaurant--Kent County, Michigan, May 2005. Morbidity and Mortality Weekly Report 55, 395-397.

Persson, K., Hamby, K., and Ugozzoli, L.A., 2005. Four-color multiplex reverse transcription polymerase chain reaction - Overcoming its limitations. Analytical Biochemistry 344, 33-42.

Peters, I.R., Helps, C.R., Hall, E.J., and Day, M.J., 2004. Real-time RT-PCR: considerations for efficient and sensitive assay design. Journal of Immunological Methods 286, 203-217.

Petitjean, J., Vabret, A., Dina, J., Gouarin, S., and Freymuth, F., 2006. Development and evaluation of a real-time RT-PCR assay on the LightCycler for the rapid detection of enterovirus in cerebrospinal fluid specimens. Journal of Clinical Virology 35, 278-284.

Pfister, T. and Wimmer, E., 2001. Polypeptide p41 of a Norwalk-like virus is a nucleic acid-independent nucleoside triphosphatase. Journal of Virology 75, 1611-1619.

Pham, T.N.Q., MacParland, S.A., Mulrooney, P.M., Cooksley, H., Naoumov, N.V., and Michalak, T.I., 2004. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. Journal of Virology 78, 5867-5874.

Pierce, K.E., Sanchez, J.A., Rice, J.E., and Wangh, L.J., 2005. Linear-After-The-Exponential (LATE)-PCR: Primer design criteria for high yields of specific single-stranded DNA and improved real-time detection. Proceedings of the National Academy of Sciences of the United States of America 102, 8609-8614.

Pierce, K.E. and Wangh, L.J., 2007. LATE-PCR and allied technologies: real-time detection strategies for rapid, reliable diagnosis from single cells. METHODS IN MOLECULAR MEDICINE 132, 47-66.

Ponka, A., Maunula, L., von Bonsdorff, C.H., and Lyytikainen, O., 1999. An outbreak of calicivirus associated with consumption of frozen raspberries. Epidemiology and Infection 123, 469-474.

Portillo, M., Fenoll, C., and Escobar, C., 2006. Evaluation of different RNA extraction methods for small quantities of plant tissue: Combined effects of reagent type and homogenization procedure on RNA quality-integrity and yield. Physiologia Plantarum 128, 1-7.

Prato, R., Lopalco, P.L., Chironna, M., Barbuti, G., Germinario, C., and Quarto, M., 2004. Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy. BMC Infectious Diseases 4.

Pruvost, M., Grange, T., and Geigl, E.M., 2005. Minimizing DNA contamination by using UNG-coupled quantitative real-time PCR on degraded DNA samples: application to ancient DNA studies. Biotechniques 38, 569-575.

Que, Y.X., Li, W., Xu, J.S., Xu, L.P., and Zhang, M.Q., 2008. A simple and versatile protocol for isolation of RNA from plant, fungi and animal. Journal of Agricultural Science and Technology 2, 63-65.

Radford, A.D., Coyne, K.P., Dawson, S., Porter, C.J., and Gaskell, R.M., 2007. Feline calicivirus. Veterinary Research 38, 319-335.

Radford, A.D., Gaskell, R.M., and Hart, C.A., 2004. Human norovirus infection and the lessons from animal caliciviruses. Current Opinion in Infectious Diseases 17, 471-478.

Reiss, R.A. and Rutz, B., 1999. Quality control PCR: A method for detecting inhibitors of Taq DNA polymerase. Biotechniques 27, 920/2-924/6.

Reuter, G., Krisztalovics, K., Vennema, H., Koopmans, M., and Szucs, G., 2005. Evidence of the etiological predominance of norovirus in gastroenteritis outbreaks - Emerging new-variant and recombinant noroviruses in Hungary. Journal of Medical Virology 76, 598-607.

Reynisson, E., Josefsen, M.H., Krause, A., and Hoorfar, J., 2006. Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR. Journal of Microbiological Methods J 66, 206-216.

Reynolds, K.A., Gerba, C.P., and Pepper, I.L., 1996. Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. Applied and Environmental Microbiology 62, 1424-1427.

Richards, G.P., Watson, M.A., and Kingsley, D.H., 2004. A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. Journal of Virological Methods 116, 63-70.

Rieht, J.A., Clements, J.E., Herzog, S., Pyper, J., Wahn, K., and Becht, H., 1993. Analysis of virus-specific RNA species and proteins in Freon-113 preparations of the Borna disease virus. Medical microbiology and immunology 182, 271-280.

Rijpens, N.P. and Herman, L.M.F., 2002. Molecular methods for identification and detection of bacterial food pathogens. Journal of Aoac International 85, 984-995.

Ringertz, O., 1971. Some aspects of the epidemiology of hepatitis in Sweden. Postgraduate Medical Journal 47, 465-472.

Rizzo, C., Di Bartolo, I., Santantonio, M., Coscia, M.F., Monno, R., De Vito, D., Ruggeri, F.M., and Rizzo, G., 2007. Epidemiological and virological investigation of a Norovirus outbreak in a resort in Puglia, Italy. BMC Infectious Diseases 7.

Rockx, B., de Wit, M., Vennema, H., Vinje, J., de Bruin, E., van Duynhoven, Y., and Koopmans, M., 2002. Natural history of human Calicivirus infection: A prospective cohort study. Clinical Infectious Diseases 35, 246-253.

Rockx, B.H.G., Vennema, H., Hoebe, C.J.P.A., Duizer, E., and Koopmans, M.P.G., 2005. Association of histoblood group antigens and susceptibility to norovirus infections. The Journal of infectious diseases 191, 749-754.

Rodriguez-Lazaro, D., Lombard, B., Smith, H., Rzezutka, A., D'Agostino, M., Helmuth, R., Schroeter, A., Malorny, B., Miko, A., Guerra, B., Davison, J., Kobilinsky, A., Hernandez, M., Bertheau, Y., and Cook, N., 2007. Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms. Trends in Food Science & Technology 18, 306-319.

Rolfe, K.J., Parmar, S., Mururi, D., Wreghitt, T.G., Jalal, H., Zhang, H., and Curran, M.D., 2007. An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. Journal of Clinical Virology 39, 318-321.

Ruiz-Palacios, G.M., Perez-Schael, I., Velazquez, F.R., Abate, H., Breuer, T., Clemens, S.A.C., Cheuvart, B., Espinoza, F., Gillard, P., and Innis, B.L., 2006. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. The New England journal of medicine 354, 11-22.

Rutjes, S.A., Italiaander, R., van den Berg, H.H.J.L., Lodder, W.J., and de Roda Husman, A.M., 2005. Isolation and detection of enterovirus RNA from large-volume water samples by using the NucliSens miniMAG system and real-time nucleic acid sequence-based amplification. Applied and Environmental Microbiology 71, 3734-3740.

Rutjes, S.A., Lodder-Verschoor, F., van der Poel, W.H.M., van Duijnhoven, Y.T.H.P., and Husman, A.M.D., 2006a. Detection of noroviruses in foods: A study on virus extraction procedures in foods implicated in outbreaks of human gastroenteritis. Journal of Food Protection 69, 1949-1956.

Rutjes, S.A., van den Berg, H.H.J.L., Lodder, W.J., and de Roda Husman, A.M., 2006b. Real-time detection of noroviruses in surface water by use of a broadly reactive nucleic acid sequence-based amplification assay. Applied and Environmental Microbiology 72, 5349-5358.

Rzezutka, A., Alotaibi, M., D'Agostino, M., and Cook, N., 2005. A centrifugation-based method for extraction of norovirus from raspberries. Journal of Food Protection 68, 1923-1925.

Rzezutka, A., Chrobocinska, M., Kaupke, A., and Mizak, B., 2008. Application of an Ultracentrifugation-based Method for Detection of Feline Calicivirus (a Norovirus Surrogate) in Experimentally Contaminated Delicatessen Meat Samples. Food Analytical Methods 1, 56-60.

Rzezutka, A., D'Agostino, M., and Cook, N., 2006. An ultracentrifugation-based approach to the detection of hepatitis A virus in soft fruits. International Journal of Food Microbiology 108, 315-320.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.T., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA. Science 239, 487-491.

Sair, A.I., D'Souza, D.H., Moe, C.L., and Jaykus, L.A., 2002. Improved detection of human enteric viruses in foods by RT-PCR. Journal of Virological Methods 100, 57-69.

Sakon, N., Yamazaki, K., Yoda, T., Kanki, M., Otake, T., and Tsukamoto, T., 2005. A norovirus outbreak of gastroenteritis linked to packed lunches. Japanese Journal of Infectious Diseases 58, 253-253.

Sala, M.R., Arias, C., Dominguez, A., Bartolome, R., and Muntada, J.M., 2009. Foodborne outbreak of gastroenteritis due to Norovirus and Vibrio parahaemolyticus. Epidemiology and Infection 137, 626-629.

Sala, M.R., Cardenosa, N., Arias, C., Llovet, T., Recasens, A., Dominguez, A., Buesa, J., and Salleras, L., 2005. An outbreak of food poisoning due to a genogroup I norovirus. Epidemiology and Infection 133, 187-191.

Sanchez, J.A., Pierce, K.E., Rice, J.E., and Wangh, L.J., 2004. Linear-After-The-Exponential (LATE) PCR: An advanced method of asymmetric PCR and its uses in quantitative real-time analysis. Proceedings of the National Academy of Sciences of the United States of America 101, 1933-1938.

Scherer, K., Johne, R., Schrader, C., Ellerbroek, L., Schulenburg, J., and Klein, G., 2010. Comparison of two extraction methods for viruses in food and application in a norovirus gastroenteritis outbreak. Journal of Virological Methods 169, 22-27.

Schmid, D., Gschiel, E., Huhulescu, S., Rupptisch, W., Böhm, G., Pichler, J., Lederer, I., Höger, G., Heuberger, S., and Allerberger, F., 2007a. Outbreak of acute gastroenteritis in an Austrian boarding school, september 2006. Eurosurveillance 12, 51-53.

Schmid, D., Stuger, H.P., Lederer, I., Pichler, A.M., Kainz-Arnfelser, G., Schreier, E., and Allerberger, F., 2007b. A foodborne norovirus outbreak due to manually prepared salad, Austria 2006. Infection 35, 232-239.

Schultz, A.C., Perelle, S., Di Pasquale, S., Kovac, K., De Medici, D., Fach, P., Sommer, H.M., and Hoorfar, J., 2010. Collaborative validation of a rapid method for efficient virus concentration in bottled water. International Journal of Food Microbiology Article in press,

Schvoerer, E., Bonnet, F., Dubois, V., Rogues, A.M., Gachie, J.P., Lafon, M.E., and Fleury, H.J., 1999. A hospital outbreak of gastroenteritis possibly related to the contamination of tap water by a small round structured virus. Journal of Hospital Infection 43, 149-154.

Schwab, K.J., Estes, M.K., Neill, F.H., and Atmar, R.L., 1997. Use of heat release and an internal RNA standard control in reverse transcription- detection of Norwalk virus from stool samples. Journal of Clinical Microbiology 35, 511-514.

Schwab, K.J. and McDevitt, J.J., 2003. Development of a PCR-enzyme immunoassay oligoprobe detection method for Toxoplasma gondii oocysts, incorporating PCR controls. Applied and Environmental Microbiology 69, 5819-5825.

Schwab, K.J., Neill, F.H., Estes, M.K., Metcalf, T.G., and Atmar, R.L., 1998. Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. Journal of Food Protection; 61, 1674-1680.

Schwab, K.J., Neill, F.H., Fankhauser, R.L., Daniels, N.A., Monroe, S.S., Bergmire-Sweat, D.A., Estes, M.K., and Atmar, R.L., 2000. Development of methods to detect "Norwalk-like viruses" (NLVs) and hepatitis A virus in delicatessen foods: Application to a food-borne NLV outbreak. Applied and Environmental Microbiology 66, 213-218.

Scipioni, A., Mauroy, A., Ziant, D., Saegerman, C., and Thiry, E., 2008a. A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition. Virology Journal 5, 94-102.

Scipioni, A., Mauroy, A., Ziant, D., Saegerman, C., and Thiry, E., 2008b. A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition. Virology Journal 5, 94-102.

Selvin, P.R., 1995. Fluorescence resonance energy transfer. Methods in enzymology 246, 300-334.

Shirato, H., Ogawa, S., Ito, H., Sato, T., Kameyama, A., Narimatsu, H., Xiaofan, Z., Miyamura, T., Wakita, T., Ishii, K., and Takeda, N., 2008. Noroviruses Distinguish between Type 1 and Type 2 Histo-Blood Group Antigens for Binding. The Journal of Virology 82, 10756-10767.

Showell, D., Sundkvist, T., Reacher, M., and Gray, J., 2007. Norovirus outbreak associated with canteen salad in Suffolk, United Kingdom. Euro Surveillance 12.

Siebenga, J.J., Vennema, H., Duizer, E., and Koopmans, M.P.G., 2007a. Gastroenteritis caused by norovirus GGII.4, the Netherlands, 1994-2005. Emerging Infectious Diseases 13, 144-146.

Siebenga, J.J., Vennema, H., Renckens, B., Bruin, E., van der Veer, B.D., Siezen, R.J., and Koopmans, M., 2007b. Epochal evolution of GGII.4 norovirus capsid proteins from 1995 to 2006. Journal of Virology 81, 9932-9941.

Siebenga, J.J., Vennema, H., Zheng, D.P., Vinje, J., Lee, B.E., Pang, X.L., Ho, E.C.M., Lim, W., Choudekar, A., Broor, S., Halperin, T., Rasool, N.B.G., Hewitt, J., Greening, G.E., Jin, M., Duan, Z.J., Lucero, Y., O'Ryan, M., Hoehne, M., Schreier, E., Ratcliff, R.M., White, P.A., Iritani, N., Reuter, G., and Koopmans, M., 2009. Norovirus Illness Is a Global Problem: Emergence and Spread of Norovirus GII.4 Variants, 2001-2007. Journal of Infectious Diseases 200, 802-812.

Sincero, T.C.M., Levin, D.B., Simões, C.M.O., and Barardi, C.R.M., 2006. Detection of hepatitis A virus (HAV) in oysters (Crassostrea gigas). Water Research 40, 895-902.

Sivapalasingam, S., Friedman, C.R., Cohen, L., and Tauxe, R.V., 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. Journal of Food Protection 67, 2342-2353.

Sobsey, M.D., Carrick, R.J., and Jensen, H.R., 1978. Improved methods for detecting enteric viruses in oysters. Applied and Environmental Microbiology 36, 121-128.

Sobsey, M.D., Wallis, C., and Melnick, J.L., 1975. Development of a simple method for concentrating enteroviruses from oysters. Applied and Environmental Microbiology 29, 21-26.

Sosnovtsev, S.V., Belliot, G., Chang, K.O., Prikhodko, V.G., Thackray, L.B., Wobus, C.E., Karst, S.M., Virgin, H.W., and Green, K.Y., 2006. Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. Journal of Virology 80, 7816-7831.

Sparkman, D.R., 1992. Paraffin wax as a vapor barrier for the PCR. Pcr-Methods and Applications 2, 180-181.

Speers, D.J., 2006. Clinical applications of molecular biology for infectious diseases. Clinical Biochemist Reviews 27, 39-51.

Stebbins, S., 2007. Rotavirus: Disease and vaccine update, 2007. Journal of Family Practice 56, S6-S11.

Stevenson, J., Hymas, W., and Hillyard, D., 2008. The use of Armored RNA as a multi-purpose internal control for RT-PCR. Journal of Virological Methods 150, 73-76.

Straub, T.M., Bentrup, K.H.Z., Orosz-Coghlan, P., Dohnalkova, A., Mayer, B.K., Bartholomew, R.A., Valdez, C.O., Bruckner-Lea, C.J., Gerba, C.P., Abbaszadegan, M., and Nickerson, C.A., 2007. In

vitro cell culture infectivity assay for human noroviruses. Emerging Infectious Diseases 13, 396-403.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., and Koopmans, M., 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in the Netherlands from 1994 through 2005. Journal of Clinical Microbiology 45, 1389-1394.

Tajiri-Utagawa, E., Hara, M., Takahashi, K., Watanabe, M., and Wakita, T., 2009. Development of a rapid high-throughput method for high-resolution melting analysis for routine detection and genotyping of noroviruses. Journal of Clinical Microbiology 47, 435-440.

Takanashi, S., Hashira, S., Matsunaga, T., Yoshida, A., Shiota, T., Tung, P.G., Khamrin, P., Okitsu, S., Mizuguchi, M., and Igarashi, T., 2009. Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis. Journal of Clinical Virology 44, 161-163.

Takkinen, J., 2006. Recent norovirus outbreaks on river and seagoing cruise ships in Europe. Euro Surveillance 11.

Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E., and Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120-131.

Taube, S., Rubin, J.R., Katpally, U., Smith, T.J., Kendall, A., Stuckey, J.A., and Wobus, C.E., 2010. High-Resolution X-Ray Structure and Functional Analysis of the Murine Norovirus 1 Capsid Protein Protruding Domain. The Journal of Virology 84, 5695-5705.

Taylor, A.E., 1996. Cardiovascular effects of environmental chemicals. Otolaryngology-Head and Neck Surgery 114, 209-211.

Tei, S., Kitajima, N., Takahashi, K., and Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. The Lancet 362, 371-373.

Terio, V., Martella, V., Moschidou, P., Di Pinto, P., Tantillo, G., and Buonavoglia, C., 2010. Norovirus in retail shellfish. Food Microbiology 27, 29-32.

Teunis, P.F.M., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., 2008. Norwalk virus: How infectious is it? Journal of Medical Virology 80, 1468-1476.

Tian, P., Brandl, M., and Mandrell, R., 2005. Porcine gastric mucin binds to recombinant norovirus particles and competitively inhibits their binding to histo blood group antigens and Caco 2 cells. Letters in Applied Microbiology 41, 315-320.

Tian, P., Engelbrektson, A., and Mandrell, R., 2008. Two-log increase in sensitivity for detection of norovirus in complex samples by concentration with porcine gastric mucin conjugated to magnetic beads. Applied and Environmental Microbiology 74, 4271-4276.

Tian, P. and Mandrell, R., 2006. Detection of norovirus capsid proteins in faecal and food samples by a real time immuno-PCR method. Journal of Applied Microbiology 100, 564-574.

Torner, N., Dominguez, A., Ruiz, L., Martinez, A., Bartolome, R., Buesa, J., and Ferrer, M.D., 2008. Acute gastroenteritis outbreaks in Catalonia, Spain: Norovirus versus Salmonella. Scandinavian Journal of Gastroenterology 43, 567-573.

Tosun, M., Ercisli, S., Karlidag, H., and Sengul, M., 2009. Characterization of Red Raspberry (Rubus idaeus L.) Genotypes for Their Physicochemical Properties. Journal of Food Science 74, C575-C579.

Traore, O., Arnal, C., Mignotte, B., Maul, A., Laveran, H., Billaudel, S., and Schwartzbrod, L., 1998. Reverse transcriptase PCR detection of astrovirus, hepatitis A virus, and poliovirus in experimentally contaminated mussels: comparison of several extraction and concentration methods. Applied and Environmental Microbiology 64, 3118-3122.

Trujillo, A.A., McCaustland, K.A., Zheng, D.P., Hadley, L.A., Vaughn, G., Adams, S.M., Ando, T., Glass, R.I., and Monroe, S.S., 2006a. Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. Journal of Clinical Microbiology 44, 1405-1412.

Trujillo, A.A., McCaustland, K.A., Zheng, D.P., Hadley, L.A., Vaughn, G., Adams, S.M., Ando, T., Glass, R.I., and Monroe, S.S., 2006b. Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. Journal of Clinical Microbiology 44, 1405-1412.

Tseng, F.C., Leon, J.S., MacCormack, J.N., Maillard, J.M., and Moe, C.L., 2007. Molecular epidemiology of norovirus gastroenteritis outbreaks in North Carolina, United States: 1995-2000. Journal of Medical Virology 79, 84-91.

Tzanetakis, I.E., Keller, K.E., and Martin, R.R., 2005. The use of reverse transcriptase for efficient first- and second-strand cDNA synthesis from single-and double-stranded RNA templates. Journal of Virological Methods 124, 73-77.

Ueki, Y., Sano, D., Watanabe, T., Akiyama, K., and Omura, T., 2005. Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. Water Research 39, 4271-4280.

Uhrbrand, K., Myrmel, M., Maunula, L., Vainio, K., Trebbien, R., N°rrung, B., and Schultz, A.C., 2010. Evaluation of a rapid method for recovery of norovirus and hepatitis A virus from oysters and blue mussels. Journal of Virological Methods 169, 70-78.

Vainio, K. and Myrmel, M., 2006. Molecular epidemiology of norovirus outbreaks in Norway during 2000 to 2005 and comparison of four norovirus real-time reverse transcriptase PCR assays. Journal of Clinical Microbiology 44, 3695-3702.

Valasek, M.A. and Repa, J.J., 2005. The power of real-time PCR. Advances in Physiology Education 29, 151-159.

van den Berg, H., Lodder, W., van der Poel, W., Vennema, H., and de Roda Husman, A.M., 2005. Genetic diversity of noroviruses in raw and treated sewage water. Research in Microbiology 156, 532-540.

van der Poel, W.H., Van der, H.R., Verschoor, F., Gelderblom, H., Vinje, J., and Koopmans, M.P., 2003. Epidemiology of Norwalk-like virus infections in cattle in The Netherlands. Vet.Microbiol. 92, 297-309.

Van Duyn, M.S. and Pivonka, E., 2000. Overview of the health benefits of fruit and vegetable consumption for the dietetics professional: Selected literature. Journal of the American Dietetic Association 100, 1511-1521.

van Duynhoven, Y.T.H.P., de Jager, C.M., Kortbeek, L.M., Vennema, H., Koopmans, M.P.G., Van Leusden, F., van der Poel, W.H.M., and Van den Broek, M.J.M., 2005. A one-year intensified study of outbreaks of gastroenteritis in The Netherlands. Epidemiology and Infection 133, 9-21.

van Maarseveen, N.M., Wessels, E., De Brouwer, C.S., Vossen, A.C.T.M., and Claas, E.C.J., 2010. Diagnosis of viral gastroenteritis by simultaneous detection of Adenovirus group F, Astrovirus, Rotavirus group A, Norovirus genogroups I and II, and Sapovirus in two internally controlled multiplex real-time PCR assays. Journal of Clinical Virology 49, 205-210.

van Zyl, W.B., Page, N.A., Grabow, W.O.K., Steele, A.D., and Taylor, M.B., 2006. Molecular epidemiology of group A rotaviruses in water sources and selected raw vegetables in southern Africa. Applied and Environmental Microbiology 72, 4554-4560.

Vasickova, P., Pavlik, I., Verani, M., and Carducci, A., 2010. Issues Concerning Survival of Viruses on Surfaces. Food and Environmental Virology 2, 24-34.

Vega, E., Smith, J., Garland, J., Matos, A., and Pillai, S.D., 2005. Variability of virus attachment patterns to butterhead lettuce. Journal of Food Protection; 68, 2112-2117.

Vennema, H., de Bruin, E., and Koopmans, M., 2002. Rational optimization of generic primers used for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction. Journal of Clinical Virology 25, 233-235.

Verhoef, L., Boxman, I.L., Duizer, E., Rutjes, S.A., Vennema, H., Friesema, I.H.M., de Roda Husman, A.M., and Koopmans, M., 2008. Multiple exposures during a norovirus outbreak on a river-cruise sailing through Europe, 2006. Euro Surveillance 12, 246-252.

Verhoef, L.P.B., Kroneman, A., van Duynhoven, Y., Boshuizen, H., van Pelt, W., and Koopmans, M., 2009. Selection Tool for Foodborne Norovirus Outbreaks. Emerging Infectious Diseases 15, 31-38.

Vernon, E., 1977. Food poisoning and salmonella infections in England and Wales, 1973-75: An analysis of reports to the Public Health Laboratory Service. Public Health 91, 225-235.

Vesikari, T., Isolauri, E., D'Hondt, E., Delem, A., Andrθ, F.E., and Zissis, G., 1984. Protection of infants against rotavirus diarrhoea by RIT 4237 attenuated bovine rotavirus strain vaccine. The Lancet 323, 977-981.

Vinje, J., Hamidjaja, R.A., and Sobsey, M.D., 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. J.Virol.Methods 116, 109-117.

Vinje, J. and Koopmans, M.P., 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. Journal of Infectious Diseases 174, 610-615.

Vinje, J., Vennema, H., Maunula, L., von Bonsdorff, C.H., Hoehne, M., Schreier, E., Richards, A., Green, J., Brown, D., Beard, S.S., Monroe, S.S., de Bruin, E., Svensson, L., and Koopmans, M.P.G., 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. Journal of Clinical Microbiology 41, 1423-1433.

Vivancos, R., Shroufi, A., Sillis, M., Aird, H., Gallimore, C.I., Myers, L., Mahgoub, H., and Nair, P., 2009. Food-related norovirus outbreak among people attending two barbeques: epidemiological, virological, and environmental investigation. International Journal of Infectious Diseases 13, 629-635.

Wadl, M., Scherer, K., Nielsen, S., Diedrich, S., Ellerbroek, L., Frank, C., Gatzer, R., Hoehne, M., Johne, R., Klein, G., Koch, J., Schulenburg, J., Thielbein, U., Stark, K., and Bernard, H., 2010. Food-borne norovirus-outbreak at a military base, Germany, 2009. BMC Infectious Diseases 10.

Wang, J.X., Jiang, X., Madore, H.P., Gray, J., Desselberger, U., Ando, T., Seto, Y., Oishi, I., Lew, J.F., Green, K.Y., and Estes, M., 1994. Sequence Diversity of Small, Round-Structured Viruses in the Norwalk Virus Group. Journal of Virology 68, 5982-5990.

Wang, Q.H., Costantini, V., and Saif, L.J., 2007. Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. Vaccine 25, 5453-5466.

Wang, Q.H., Souza, M., Funk, J.A., Zhang, W., and Saif, L.J., 2006. Prevalence of noroviruses and sapoviruses in swine of various ages determined by reverse transcription-PCR and microwell hybridization assays. Journal of Clinical Microbiology 44, 2057-2062.

Watanabe, M., Kohdera, U., Kino, M., Haruta, T., Nukuzuma, S., Suga, T., Akiyoshi, K., Ito, M., Suga, S., and Komada, Y., 2005. Detection of adenovirus DNA in clinical samples by SYBR Green real-time polymerase chain reaction assay. Pediatrics International 47, 286-291.

Webby, R.J., Carville, K.S., Kirk, M.D., Greening, G., Ratcliff, R.M., Crerar, S.K., Dempsey, K., Sarna, M., Stafford, R., Patel, M., and Hall, G., 2007. Internationally distributed frozen oyster meat causing multiple outbreaks of norovirus infection in Australia. Clinical Infectious Diseases 44, 1026-1031.

Weiss, C. and Clark, H.F., 1985. Rapid inactivation of rotaviruses by exposure to acid buffer or acidic gastric juice. Journal of General Virology 66, 2725-2730.

Wernars, K. and Notermans, S., 1990. Gene probes for detection of food-borne pathogens. Gene Probes for Bacteria 353-388.

White, L.J., Ball, J.M., Hardy, M.E., Tanaka, T.N., Kitamoto, N., and Estes, M.K., 1996. Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. Journal of Virology 70, 6589-6597.

Widdowson, M.A., Sulka, A., Bulens, S.N., Beard, R.S., Chaves, S.S., Hammond, R., Salehi, E.D.P., Swanson, E., Totaro, J., Woron, R., Mead, P.S., Bresee, J.S., Monroe, S.S., and Glass, R.I., 2005. Norovirus and foodborne disease, United States, 1991-2000. Emerging Infectious Diseases 11, 95-102.

Wilson, I.G., 1997. Inhibition and Facilitation of Nucleic Acid Amplification. Applied and Environmental Microbiology 63, 3741-3751.

Winn-Deen, E.S., 1998. Direct fluorescence detection of allele-specific PCR products using novel energy-transfer labeled primers*. Molecular Diagnosis 3, 217-222.

Witlox, K.J., Nguyen, T.N., Bruggink, L.D., Catton, M.G., and Marshall, J.A., 2008. A comparative evaluation of the sensitivity of two automated and two manual nucleic acid extraction methods for the detection of norovirus by RT-PCR. Journal of Virological Methods 150, 70-72.

Wittwer, C.T., Reed, G.H., Gundry, C.N., Vandersteen, J.G., and Pryor, R.J., 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. Clinical Chemistry 49, 853-860.

Wobus, C.E., Thackray, L.B., and Virgin, H.W., 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. Journal of Virology 80, 5104-5112.

Wolf, S., Hewitt, J., and Greening, G.E., 2010. Viral Multiplex Quantitative PCR Assays for Tracking Sources of Fecal Contamination. Applied and Environmental Microbiology 76, 1388-1394.

Wolf, S., Williamson, W.M., Hewitt, J., Rivera-Aban, M., Lin, S., Ball, A., Scholes, P., and Greening, G.E., 2007. Sensitive multiplex real-time reverse transcription-PCR assay for the detection of human and animal noroviruses in clinical and environmental samples. Applied and Environmental Microbiology 73, 5464-5470.

Worm, H.C., van der Poel, W.H.M., and BrandstΣtter, G., 2002. Hepatitis E: an overview. Microbes and infection 4, 657-666.

Xi, J.A., Graham, D.Y., Wang, K.N., and Estes, M.K., 1990. Norwalk Virus Genome Cloning and Characterization. Science 250, 1580-1583.

Yamashita, T., Sakae, K., Ishihara, Y., Isomura, S., and Utagawa, E., 1993. Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. Journal of Clinical Microbiology 31, 2938-2943.

Yan, H.N., Yagyu, F., Okitsu, S., Nishio, O., and Ushijima, H., 2003. Detection of norovirus (GI, GII), Sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. Journal of Virological Methods 114, 37-44.

Ye, W., Liu, L., Zheng, W., Lin, J., and Yin, J., 2009. Comparison of RNA extraction methods applied to gene cloning of the taxol-producing fungi. African Journal of Microbiology Research 3, 632-636.

Yeh, H.Y., Hwang, Y.C., Yates, M.V., Mulchandani, A., and Chen, W., 2008. Detection of hepatitis A virus by using a combined cell culture-molecular beacon assay. Applied and Environmental Microbiology 74, 2239-2243.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I., and Tsukamoto, T., 2007. Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. Journal of Medical Virology 79, 326-334.

Yoneyama, T., Kiyohara, T., Shimasaki, N., Kobayashi, G., Ota, Y., Notomi, T., Totsuka, A., and Wakita, T., 2007. Rapid and real-time detection of hepatitis A virus by reverse transcription loop-mediated isothermal amplification assay. Journal of Virological Methods 145, 162-168.

Zhang, J. and Byrne, C.D., 1999. Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR. Biochemical Journal 337, 231-241.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I., and Monroe, S.S., 2006. Norovirus classification and proposed strain nomenclature. Virology 346, 312-323.

Zheng, D.P., Widdowson, M.A., Glass, R.I., and Vinje, J., 2009. Molecular Epidemiology of GII. 4 Noroviruses in the US between 1994 and 2006. Journal of Clinical Microbiology, In press.

Zipper, H., Brunner, H., Bernhagen, J., and Vitzthum, F., 2004. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Research 32, e103-e113.

Zomer, T.P., De Jong, B., Kühlmann-Berenzon, S., Nyrén, O., Svenungsson, B., Hedlund, K.O., Ancker, C., Wahl, T., and Andersson, Y., 2009. A norovirus outbreak at a manufacturing company. Epidemiology and Infection 138, 501-506.

Curriculum vitae

CURRICULUM VITAE

Ambroos Stals werd geboren op 19 mei 1984 te Gent. In 2002 behaalde hij het diploma Wetenschappen-Wiskunde (6u) aan Koninklijk Atheneum III te Gent en in 2006 behaalde hij met onderscheiding het diploma Licentiaat in de Biotechnologie aan de Universiteit Gent.

Sinds februari 2007 is hij werkzaam als wetenschappelijk medewerker aan de eenheid Technologie & Voeding van het Instituut voor Landbouw- en Visserijonderzoek (ILVO) en het Laboratorium voor Levensmiddelenmicrobiologie en –conservering (Faculteit Bio-ingenieurswetenschappen, Universiteit Gent) in het kader van het Belspo project genaamd NORISK, “Transmissieroutes van norovirussen, opduikende humane pathogenen aanwezig in de voedselketen” (project no: SSD - NORISK - SD/AF/01). Hij werkte aan dit doctoraat onder leiding van zijn promotoren prof. dr. ir. Mieke Uyttendaele (Universiteit Gent) en dr. ir. Els Van Coillie (ILVO).

Tijdens zijn onderzoek publiceerde hij in internationale tijdschriften, en nam deel aan internationale en nationale workshops, symposia en congressen. Verder begeleidde hij een thesisstudente en werkte hij mee aan de practica voor de vakken ‘Moleculair-microbiële technieken’ in de opleiding Bio-ingenieur.

CURRICULUM VITAE

Ambroos Stals was born in Ghent on May the 19th in 1984. In 2002 he obtained a degree in Science and Mathematics at Koninklijk Atheneum III in Ghent. In 2006 he graduated cum laude as master in Biotechnology at the University of Ghent.

He has been working since February 2007 as research associate at the Technology and Food Science Unit of the Institute for Agricultural and Fisheries Research (ILVO) and at the Laboratory of Food Safety and Food Preservation at the Faculty of Bioscience Engineering, University of Ghent. He performed his PhD study within the Belspo project named NORISK, "Transmission routes of noroviruses, emerging human pathogens in food (project no: SSD - NORISK - SD/AF/01). Promotors of this PhD study were prof. dr. ir. Mieke Uyttendaele (University of Ghent) en dr. ir. Els Van Coillie (ILVO).

During his PhD study she published in several international journals, attended different national and international workshops, symposia and congresses. He also guided a thesis student and was involved with practical sessions of the course 'Moleculair-microbiële technieken' for the Master Bioscience Engineering.

PUBLICATION LIST

Publications in A1 peer-reviewed journals

- Ambroos Stals, Hadewig Werbrouck, Leen Baert, Nadine Botteldoorn, Lieve Herman,Mieke Uyttendaele and Els Van Coillie.
  Laboratory efforts to eliminate contamination problems in the real-time RT-PCR detection of noroviruses.
  Journal of Microbiological Methods 77, 72–76 (2009).

- Ambroos Stals, Leen Baert, Nadine Botteldoorn, HadewigWerbrouck, Lieve Herman, Mieke Uyttendaele and Els Van Coillie.
  Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1.
  Journal of Virological Methods 161, 247–253 (2009).

- Ambroos Stals, Leen Baert, Els Van Coillie and Mieke Uyttendaele.
  Evaluation of a norovirus detection methodology for soft red fruits
  Food Microbiology 28 (1), 52-58 (2011).

- Ambroos Stals, Leen Baert, Vicky Jasson, Els Van Coillie and Mieke Uyttendaele.
  Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results.
  Journal of Food protection, In press.

- Ambroos Stals, , Ann De Keuckelaere, Els Van Coillie and Mieke Uyttendaele.
  Evaluation of a norovirus detection methodology for ready-to-eat foods.
  International Journal of Food Microbiology, In press.

- Leen Baert, Mieke Uyttendaele, Ambroos Stals, Els Van Coillie, Katelijne Dierick, Johan Debevere and Nadine Botteldoorn.
  Reported foodborne outbreaks due to noroviruses in Belgium during 2007: the link between food and patient investigations in an international context.
  Epidemiology and Infection 137, 316-325 (2008).

Awards

- "Kemin Young Scientist Award for Innovation in the Food & Health Chain" at the Re$earch seminar. Belgium (Ghent), 24/02/2009.
  Ambroos Stals, Hadewig Werbrouck, Leen Baert, Nadine Botteldoorn, Elke Wollants, Lieve Herman,Mieke Uyttendaele and Els Van Coillie.
  Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1.

Oral presentations

- Ambroos Stals, Hadewig Werbrouck, Leen Baert, Nadine Botteldoorn, Elke Wollants, Lieve Herman,Mieke Uyttendaele and Els Van Coillie.
  Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1.
  - Rapid Methods Europe 2009. The Netherlands (Noordwijk), 26-28/01/2009.
  - KVCV - Trends in food analysis VI. Belgium (Ghent), 19/05/2009.

- Ambroos Stals, Leen Baert, Vicky Jasson, Els Van Coillie and Mieke Uyttendaele.
  Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results.
  - 2nd COST 929 symposium. Turkey (Istanbul), 7-9/10/2010.

- Ambroos Stals, Leen Baert, Ann De Keuckelaere, Els Van Coillie and Mieke Uyttendaele.
  Evaluation of a norovirus detection methodology for ready-to-eat foods.
  - 2nd COST 929 symposium. Turkey (Istanbul), 7-9/10/2010.

- Ambroos Stals.
  Detection of noroviruses in food.
  - Workshop "snelle methoden in de levensmiddelenmicrobiologie". Belgium (Ghent), 18/03/2010.

Extended abstracts of symposia and workshops

- Ambroos Stals, Hadewig Werbrouck, Leen Baert, Nadine Botteldoorn, Elke Wollants, Lieve Herman,Mieke Uyttendaele and Els Van Coillie.
  Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1.
  Poster and abstract (in book of abstracts):
  - 14th PhD symposium on Applied Biological Sciences. Belgium (Ghent), 14/09/2008.
  - Benelux qPCR symposium, poster and abstract. Belgium (Ghent), 06/10/2008.
  - 1st COST929 Symposium "Current Developments in Food and Environmental Virology". Italy (Pisa), 9-11/10/2008.
  - Rapid Methods Europe 2009. The Netherlands (Noordwijk), 26-28/01/2009.
  - KVCV - Trends in food analysis VI. Belgium (Ghent), 19/05/2009.
  - Re$earch seminar. Belgium (Ghent), 24/02/2009.

- Ambroos Stals, Leen Baert, Els Van Coillie and Mieke Uyttendaele.
  Evaluation of a norovirus detection methodology for soft red fruits
  Poster and abstract (in book of abstracts):
  - 14th conference on food microbiology. Belgium (Liège), 18-19/09/2009.
  - 2009 IAFP European Symposium. Germany (Berlin), 7-9/10/2009

- Ambroos Stals, Leen Baert, Ann De Keuckelaere, Els Van Coillie and Mieke Uyttendaele.
  Evaluation of a norovirus detection methodology for ready-to-eat foods.
  Poster and abstract (in book of abstracts):
  - 15th conference on food microbiology. Belgium (Liège), 16-17/09/2010.
  - Exchange 2010. Belgium (Ghent), 28/09/2010
  - 4th international conference on Caliciviruses. Chili (Santa Cruz), 16-19/10/2010.
  - 2nd COST 929 symposium. Turkey (Istanbul), 7-9/10/2010.

- Ambroos Stals, Leen Baert, Vicky Jasson, Els Van Coillie and Mieke Uyttendaele. Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results.
    - Food Micro 2010. Denmark (Copenhagen), 30/08/2010 – 03/09/2010.
    - 15th conference on food microbiology. Belgium (Liège), 16-17/09/2010.
    - Exchange 2010. Belgium (Ghent), 28/09/2010
    - 4th international conference on Caliciviruses. Chili (Santa Cruz), 16-19/10/2010.
    - 2nd COST 929 symposium. Turkey (Istanbul), 7-9/10/2010.

- Nadine Botteldoorn, Leen Baert, Ambroos Stals, Els Van Coillie, Katelijne Dierick, Johan Debevere, Mieke Uyttendaele.
  Norovirus detection in food-borne outbreaks: linking food and patient results,
    - 13th conference on food microbiology. Belgium (Liège), 11-12/09/2008
    - Food Micro 2008. Scotland (Aberdeen), 1-4/09/2008.